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EXPRESSION AND FUNCTIONS OF IL-24 AND SOCS3 IN *PSEUDOMONAS AERUGINOSA* KERATITIS IN A C57BL/6 MOUSE MODEL

by

BING XU

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2016

MAJOR: ANATOMY AND CELL BIOLOGY

Approved By:

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Advisor Date

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DEDICATION

To William James Ross IV

Thank you for walking with me through life's ups and downs.

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ACKNOWLEDGEMENTS

First and foremost, I thank my advisor and mentor, Dr. Fu-shin X. Yu. He put great patience and time to my scientific training in the past four years. He was always available whenever I ran into a problem. He would analyze the problem, pinpoint the key issues, and come out a possible solution with me. At the same time, he gave me the freedom to figure out the problems myself and explore all possibilities. For that, I will always be thankful.

I would like to thank my committee members: Dr. Ashok Kumar, Dr. Jena Steinle, and Dr. Theodore J. Standiford. Their questions guided me to think deeper and form a global picture of my project. They helped me to develop critical thinking on every subject. Specially, I would like to thank Dr. Paul. D. Walker. I could not ask for a better graduate director. He was always available to talk about all the troubles throughout this entire process. His sense of humor made me see the hope in the dark days. I would like to think our department chair Dr. Linda Hazlett for her support to graduate students. I would like to thank Selina, Terri, Tonia, and Rose in the office for all the hard work to make things convenient for us.

I would like to thank my lab members: Nan Gao, Haijing Sun, Patric Lee, Xinhan Cui, Jiayin Wu and Chenxi Yan for their help in everyday experiments. I specially thank Nan Gao and Haijing Sun for teaching me all the experiment techniques and troubleshooting with me.

Finally, I would like to thank my family for all the support in these years. They made everything possible for me. I would like to thank all my friends. Life is so much fun with friends. Lastly, I thank my husband for giving me strength and happiness.

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INTRODUCTION

The structure and functions of the cornea

The human eye is a sensory organ, which detects light waves and gives us the sense of sight, through which we can observe and learn the surrounding world. It is estimated that about 80% of information we receive comes from the sight. The eye works as a camera. The incoming light waves from an object enter the eye through the cornea, which acts as a lens to refract the light. The light continues to pass through a number of tissues, as it passes through the inner components of a camera. The light is eventually detected by the retina, which is equivalent to the film of a camera, and converted into electrical signals to transmit to the brain, where it is interpreted or “seen” as a visual image.

The cornea is the clear, dome-shaped structure at the front of the eye. It is named the “window” of the sight. It is covered by the tear film and physically protected by the eyelids in the front. The cornea is structurally divided into five layers: the epithelium, Bowman's layer in human and the basement membrane in rodents and other animals, the stroma, Descemet's membrane, and the endothelium (Fig. 1). The cornea is transparent and devoid of blood vessels, and is well known as an immune-privileged site in the human body. Disruption of the structure, for example, vascularization or scar formation, would cause the cornea to lose its transparency, leading to visual impairment. The cornea is extremely abundant in free nerve endings and exquisitely sensitive to foreign objects. Even the smallest piece of dust on the cornea would cause significant discomfort and irritation.

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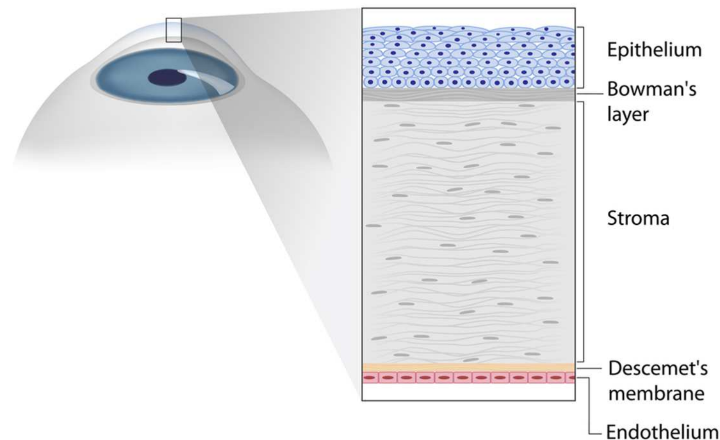

Figure 1 The structure of the cornea. The cornea can be divided into five layers. From anterior to posterior: epithelial layer, Bowman's layer, stromal layer, Descemet's layer, and endothelial layer. Open access image provided by Discoveryeye. http://discoveryeye.org/corneal-transplant-surgery-terms-defined/

The corneal epithelium is the stratified epithelium consisting 5-7 layers of cells covering the cornea and forms the first physical, chemical, and biological barrier of the eye against external environment. The tear film on the epithelial surface contains lysozyme, lactoferrin, IgA, etc., which have bacteriostatic or bactericidal activity (Ueta and Kinoshita, 2010). The membrane-tethered mucins, including MUC1, MUC4, and MUC16, form a dense glycocalyx to serve as a physical barrier to prevent bacterial adherence on the surface of corneal epithelium (Govindarajan and Gipson, 2010). The apical layer of the stratified corneal epithelium possesses tight junctions, the most important biological barrier against microbial infection and chemical insults. However, if this barrier function is compromised, the cornea is susceptible to various insults. In response to microbial insults, corneal epithelial cells can produce antimicrobial peptides,

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including β-defensins, cathelicidin (LL37), and calprotectin (the heterodimer S100A8 and A9), etc. to restrict the availability of nutrition to microbes and/or to kill microbes directly. Recently, a novel class of antimicrobial peptides has been identified that are processed from cytokeratin-6A in corneal epithelial cells (Tam et al., 2012). These keratin-derived antimicrobial peptides (KDAMPs) can bind to bacteria directly and induce permeabilization. They have a broad spectrum of anti-microbial activity.

The Bowman's membrane is a smooth, acellular collagen layer, which serves as a barrier between the epithelium and the stroma to prevent direct traumatic insults in the corneal stroma. The stroma takes up to 90% of the corneal thickness and consists of parallel collagen fibers with sparsely embedded keratinocytes. Healthy corneal epithelium and stroma contain resident immune cells, including heterogeneous macrophages and dendritic cells (Brissette-Storkus et al., 2002; Chinnery et al., 2008; Chinnery et al., 2007; Hamrah and Dana, 2007; Hamrah et al., 2003) (Fig. 2). These cells initiate host response to pathogens and secrete cytokines and chemokines to recruit immune cell infiltration to mediate pathogen killing as well as inflammatory responses. The Descemet's membrane is a thin acelluar layer that acts as the modified basement membrane of the endothelium. The endothelium is a single cellular layer responsible for the transportation of fluid and solutes between the aqueous and the stroma, maintaining the hydration of the stroma.

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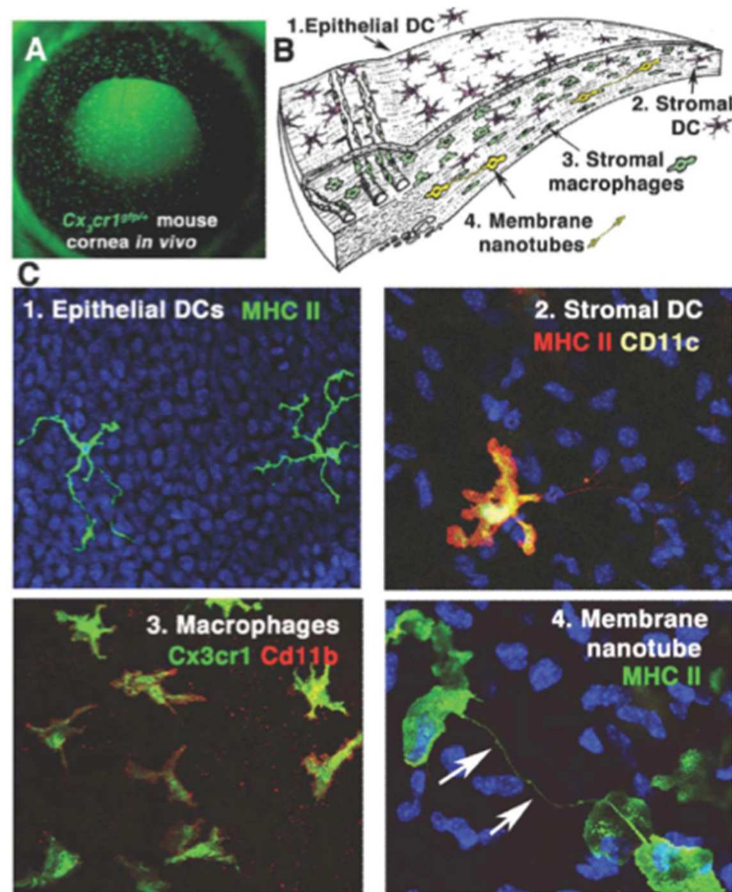

Figure 2 Resident immune cells in the cornea. (A) Corneal image from CX_3CR1^{GFP+} mouse showing normal cornea is populated with resident bone marrow derived cells. (B) Schematic graph showing the distribution of dendritic cells (DC) in both the epithelial and stromal layer, and macrophages in the stromal layer. (C) Mouse corneal whole mount images in panels 1-4 illustrating each cell population in Fig. 1B. Panel 1: Wild type cornea incubated with MHC II antibody demonstrating the expression of epithelial DCs. Panel 2: Cornea from CD11c eYFP mouse incubated with MHC II antibody demonstrating the expression of stromal DC**.** Panel 3: Cornea from CX_3CR1^{GFP+} mouse incubated with CD11b antibody demonstrating the expression of macrophages in the stroma. Panel 4: Wild type cornea incubated with MHC II antibody demonstrating the presence of membrane nanotube between MHC II+ cells. Images obtained from NIH Public Access manuscript (Pearlman et al., 2013)

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Infectious keratitis

Infectious keratitis is caused by pathogens including bacteria, viruses, fungi, and protozoa to invade the cornea and cause abrasion and loss of corneal epithelium, with inflammation in the underlying stroma (Keay et al., 2006). Infectious keratitis is also named as “ulcerative keratitis” because of its characteristic defects in the corneal epithelium. Clinical signs and symptoms include inflammation, irritation, and discharge. The infected cornea appears to be red and swollen. The inflammation causes the cornea to lose its transparency, presenting as corneal opacity. Since the cornea is richly innervated, the irritation of the cornea causes copious tear production, sensitivity to light, and extremely pain and discomfort. The diseased pathogens, epithelial cells, and infiltrating cells (mainly neutrophils) are presented as discharge. Frequently, certain pathogens have their specific presentations other than the general signs and symptoms. Infectious keratitis is a medical emergency that requires prompt diagnosis and treatment. The induced inflammation would eventually cause neovascularization and scaring in the cornea, leading to vision impairment or loss.

Predisposing factors of infectious keratitis

The incidence of infectious keratitis varies among different reports, from 6.3 to 11 per 100, 000 populations per year (Erie et al., 1993; Lam et al., 2002). The prevalence and the spectrum of causative pathogens largely depend on climate and predisposing factors. While *Candida* is the predominant fungal species in temperate climates (Ritterband et al., 2006; Tanure et al., 2000; Tuft and Tullo, 2009), filamentous fungi account for the majority of fungal keratitis in tropical or subtropical areas, with *Fusarium* and *Aspergillus* as prevalent fungi, respectively (Chander and Sharma, 1994; Houang et

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al., 2001; Liesegang and Forster, 1980; Upadhyay et al., 2001; Wong et al., 1997). Infectious keratitis caused by other etiological agents, however, does not correlate with geographical location or environmental factors in such an obvious way. Intact cornea of a healthy individual is extremely resistant to infection. Majority of patients (87%) have at least one predisposing factor when the infection occurs (Pachigolla et al., 2007). Risk factors include ocular trauma, extended contact lens wear, chronic ocular surface disease, ocular surgery, or systemic immune compromised diseases, etc. (Bourcier et al., 2003; Dart et al., 1991; Fong et al., 2004; Keay et al., 2006; Wong et al., 2003). Ocular trauma and extended contact lens wear are the two most preventable risk factors in young age population (Bourcier et al., 2003; Keay et al., 2006).

Contact lens as a major predisposing factor

As people are enjoying the convenience and cosmetic effects brought by contact lens, infections keratitis accompanies the benefits, and continues to be problematic despite decades of research. Contact lens wear is the predominant risk factor for infectious keratitis in developed counties (Keay et al., 2006; Schein et al., 2005). Multiple reports have proved that lens material is not an independent risk factor (Dart et al., 2008; Stapleton et al., 2008). Poor hygiene and extended wear are the two major risk factors (Dart et al., 1991; Keay et al., 2009; Schein et al., 1989). During contact lens wear, a number of factors would increase the chance of microbial contamination on ocular surface, including microbe entry through outside air environment, finger contact with lens, contact lens, care solution, and storage case. Risks factors include infrequent lens disinfection or case cleaning, hand contamination before handling lens, self-poor general hygiene, and lower socioeconomic class (Dart et al., 2008; Dart et al., 1991;

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Keay et al., 2009; Radford et al., 1998; Stapleton et al., 2008). It is well known that contamination does not always end up with infection. Contamination is necessary for infection, but it is not sufficient. Infection further requires the adaptation of microbes and the physiological changes of corneal response.

Extended contact lens wear fulfills these other two requirements, and hence becomes the most significant risk factor. It is reported that infection occurs in one week after *P. aeruginosa*-contaminated lens being placed on a rat cornea (Tam et al., 2010). Further research demonstrates that extended wear facilitates microbe growth and formation of biofilms on the posterior side of lens surface (Tam et al., 2010). The biofilms foster microbial adaptation on corneal surface and render microbes more resistant to disinfectant or antibiotics. More importantly, during the extended “incubation time” with corneal epithelial cells, microbes are able to alter gene expression to enhance virulent factors to invade corneal epithelial cells (Fleiszig and Evans, 2010). Therefore, extended wear allows microbes to gain more time to prepare them to penetrate host cells.

On the other hand, extended contact lens wear alters the defensive ability of corneal epithelial cells against microbial invasion. It is reported that after extended contact lens wear (3d), *in vitro* cultured corneal epithelial cells lost their ability to up-regulate antimicrobial peptides, such as human β-defensin-2 (hBD2), when they were challenged with bacterial components (Maltseva et al., 2007; Mun et al., 2009). The tear film contributes to corneal defense against infection (Fleiszig et al., 2003; Kwong et al., 2007; McNamara et al., 2005). Contact lens being placed on the cornea may disrupt tear biochemistry on the ocular surface (Fleiszig and Evans, 2010). The tear film may

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be excluded out the corneal surface if contact lens is placed too close. The placement of contact lens may disrupt or shut down tear exchange between anterior and posterior lens compartments (Paugh et al., 2001). During extended wear, the lack of tear exchange may hamper the clearance of microbes on the corneal surface, as demonstrated by more infectious keratitis has been found in soft lens wearers than rigid gas-permeable lens wearer since soft lens permits less tear exchange (McNamara et al., 1999). In addition, long-term contact lens wear inhibits normal shedding of corneal epithelial cells (Cavanagh et al., 2002), causes corneal epithelial thinning (Cavanagh et al., 2002), and leads to the formation of membrane lipid rafts on corneal epithelial cells (Robertson et al., 2007). Lipid rafts would enhance the internalization of bacteria in corneal epithelial cells. Extended contact lens wear also induces a hypoxic environment in corneal epithelial cells, thereby increasing bacterial invasion and inflammatory cytokine activation (Zaidi et al., 2004). Therefore, contact lens wear becomes a significant risk factor for corneal infection.

P. aeruginosa (PA)

There are 80-140 million contact lens wearers around the world, presenting a significant risk for microbial keratitis (Stapleton et al., 2007). *PA* remains the predominant isolated pathogen associated with contact lens related keratitis (Stapleton and Carnt, 2012). The strong correlation between *PA* and contact lens-related keratitis is intriguing. As a matter of fact, before the invention of contact lens, *PA* was a rare isolate from microbial keratitis. A battle of research undergoes to explore the reasons why *PA* can preferably exploit the niche created by contact lens.

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One of the most important factors is *PA* has a large genome size. The genome of *PA* strain *PAO1* has been completely sequenced and contains 6.3 million base pairs, the largest bacterial genome sequenced so far (Stover et al., 2000) (Fig. 3). The genome has 5, 570 predicted open reading frames (ORFs), denoting its genetic complexity (Stover et al., 2000). *PA* has the largest proportion of genes devoted to the regulatory system, for example, transcriptional regulators and environmental sensors, permitting it to adapt to and thrive in diverse environments (Stover et al., 2000). In addition, *PA* has the capacity to metabolize a wide variety of organic substrates, contributing to its enzymatic modification and degradation of antibiotics (Stover et al., 2000). Specially, *PA* has four multidrug efflux systems that are members of resistance-nodulation-cell division (RND) family, which confer the intrinsic resistance of *PA* to antibiotics and disinfectants (Nikaido, 1998; Westbrock-Wadman et al., 1999). The size and complexity of *PA* genome reflex its evolutionary adaptation to diverse environment and resistance to antimicrobial substances.

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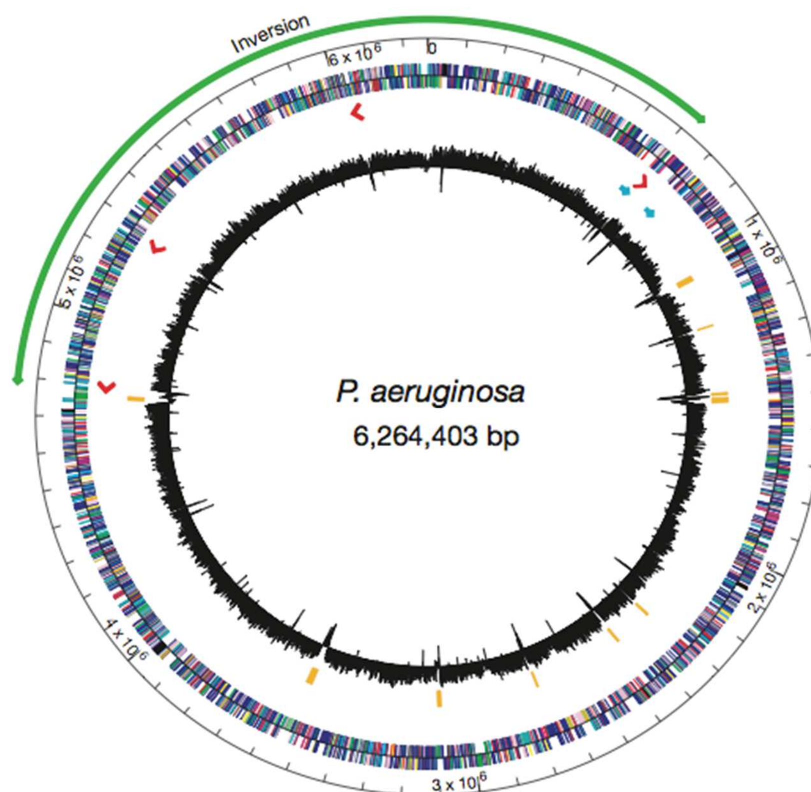

Figure 3 The genome of *P. aeruginosa* strain *PAO1*. Each tick in the outermost circle represents 100kb. *PAO1* contains a total of 6,264,403 bp genes. The genes are color-coded according to their functional categories and direction of transcription. The outer band is the plus strand; the inner band is the minus strand. Detailed description can be found in the cited article. (Stover et al., 2000)

Virulence factors play important roles in *PA* invasion and infection. A key mechanism that *PA* uses to control the expression of virulence factors is through quorum sensing (QS). Quorum sensing is a global gene regulatory system that organisms use to respond to surrounding environment (Willcox et al., 2008). Organisms secrete signaling molecules to surrounding environment. When a certain threshold concentration is reached, the molecules can be detected by specific receptor proteins to

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initiate signal transduction and induce QS-controlled gene expression. It is reported that as many as 10% of *PA* genome is controlled by QS (Schuster et al., 2003; Wagner et al., 2003; Willcox et al., 2008). Many of these genes directly or indirectly mediate the pathogenesis of *PA* and its persistence in host cells (Fig. 4). The acylated homoserine lactone (AHL) QS system seems to be routinely used by a variety of pathogenic Gram-negative bacteria, including *PA* (Willcox et al., 2008). Two hierarchical QS circuits exist in *PA* to respond to AHL signals: the primary Las system (Gambello and Iglewski, 1991) and secondary Rhl system (Brint and Ohman, 1995; Pearson et al., 1997). Las system encodes the proteins LasI and LasR (Gambello and Iglewski, 1991). LasI has catalytic ability and LasR is a DNA-binding transcription regulator. LasI mediates the production of the AHL molecule N-3-oxododecanoyl-L-homoserine lactone (3O- C12-HSL) (Pearson et al., 1994), which, accompanied by LasR, binds to the promoters of QS-control genes to regulate virulence factor expression, such as lasB (elastase), aprA (alkaline protease), and eoxA (exotoxin A) (Chapon-Herve et al., 1997; Gambello and Iglewski, 1991; Gambello et al., 1993; Passador et al., 1993; Pessi and Haas, 2000; Seed et al., 1995; Storey et al., 1998; Toder et al., 1994; Toder et al., 1991). The expression of QS-regulated genes can positively feedback to the Las circuit to induce more AHL production, and in the meantime, induce a secondary QS circuit, the Rhl system (Pearson et al., 1995; Seed et al., 1995). The Rhl system consists of RhlI and the receptor, RhlR (Brint and Ohman, 1995; Ochsner et al., 1994; Pearson et al., 1995; Pearson et al., 1997). RhlI synthesizes N- butyryl-L-homoserine lactone (C4-HSL), which can bind to RhIR when the concentration reaches a certain threshold to induce the expression of QS-control genes, including rhlAB (rhamnolipid synthesis genes),

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rpoS (the stationary-phase sigma factor), lecA (type-1 lectin), lecB (type-II lectin), and genes involved in pyocyanin production, et al. (Brint and Ohman, 1995; Latifi et al., 1995; Ochsner et al., 1994; Pearson et al., 1997; Pessi and Haas, 2000; Winzer et al., 2000). In addition, there is a third *PA* QS signal, the Pseudomonas quinolone signal (PQS), which senses the balanced production of 3O-C12-HSL and C4-HSL (Pesci et al., 1999). The production and bioactivity of PQS intimately depend on both the Las and Rhl QS systems (McGrath et al., 2004). The PQS can mediate the transcription of Rhl-dependent *PA* virulence genes, such as pyocyanin and rhamnolipid (Deziel et al., 2004). Thus, QS controls an arsenal of virulence gene expression to facilitate *PA* pathogenesis.

Virulence factors of *PA*

PA is ubiquitous in environment and may be present in ocular surface in healthy individuals. The physio-chemical characteristics of bacterial surface often influence its adhesive ability. Bacteria with hydrophobic surface tend to adhere better than hydrophilic bacteria. It is reported that *PA* GSU#3 has a surface water contact angle of 132 degrees, which is much higher than that of *S. aureus* strains (around 20-36 degrees), demonstrating the hydrophobicity of *PA* (Bruinsma et al., 2001; Klotz et al., 1989). This prominent phenomenon contributes to its adhesion and colonization to contact lens and storage case.

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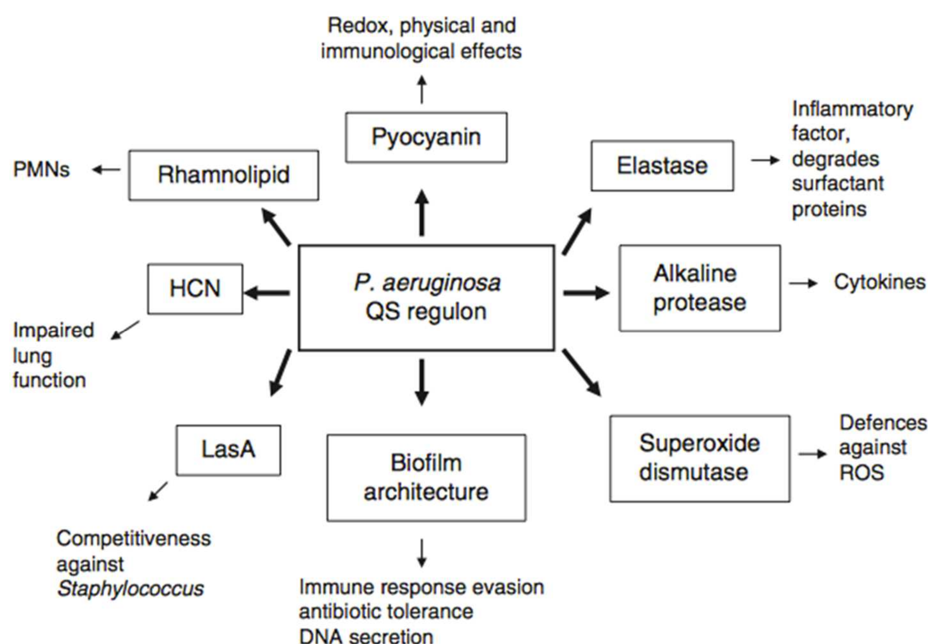

Figure 4 *PA* virulence factors regulated by quorum sensing (QS) system. ROS: reactive oxygen species. PMN: polymorphonuclear leukocyte. (Winstanley and Fothergill, 2009)

Type Three Secretion System (TTSS) is a protein appendage found in many pathogenic Gram-negative bacteria, including *PA*. The structure of TTSS consists of two parts: the base and the needle (Fig. 5). The base anchors in the membranes of Gram-negative bacteria; the needle starts at the cytoplasm of the bacteria, passes through the inner and outer membranes and protrudes from the cells (Cornelis, 2006; Moraes et al., 2008). The needle helps bacteria to detect the presence of eukaryotic cells. Once the needle is inserted into eukaryotic membrane, the effector proteins from TTSS would pass through the needle and be secreted directly into eukaryotic cells, where they exert a number of effects. Four effector proteins have been identified so far: exoS, exoU, exoY, and ExoT. Based on the presence of particular effector proteins of TTSS, *PA* strains can be divided into two subgroups: invasive stains and cytotoxic strains,

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expressing exoenzymes S (exoS) and U (exoU), respectively (Stapleton and Carnt, 2012). *PA* with exoS invades epithelial cells, proliferates inside the cells, and induces cell death through the disruption of host cell cytoskeleton (Barbieri and Sun, 2004). *PA* expressing exoU proliferates extracellularly. ExoU is a potent cytotoxin that can directly cause damage to epithelial cell membrane, and induce a cascade of inflammation (Sato and Frank, 2004). Therefore, TTSS plays an important role in the pathogenesis of *PA*.

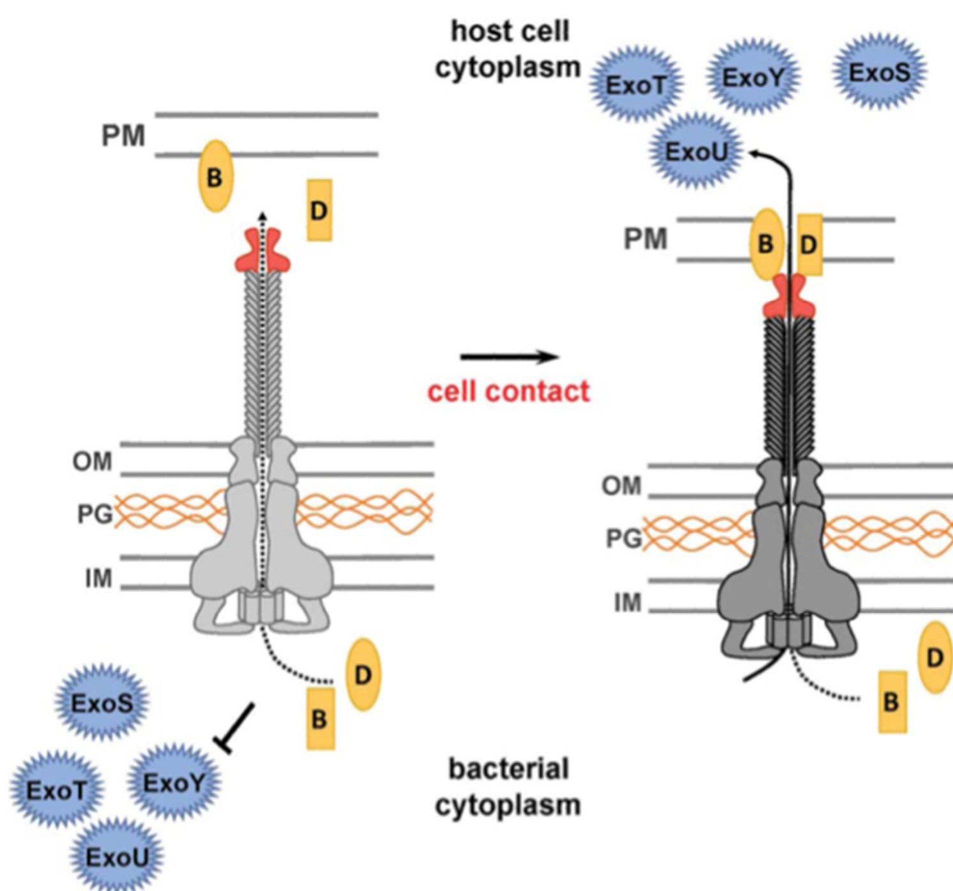

Figure 5 Structure of the Type Three Secretion System (TTSS) of *PA*. TTSS consists of a base and a needle complex. When the needle complex detects and anchors to host cells, effector toxins including ExoS, ExoT, ExoY, and ExoU would be injected directly into the host cells through the needle complex. (Pearlman et al., 2013)

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Flagellum is the major motility element of *PA*. Each *PA* cell has a single flagellum, localizing at a cell pole. Flagellum provides bursts of straight line swimming motions with occasional “tumbles” for bacterium to reorient itself. It also functions as an adhesin and inflammation initiator related to the pathogenesis of *PA* (Miao et al., 2007). Flagellum is mainly composed of flagellin, a globular protein that arranges itself helically to form a hollow cylinder. The protein flagellin is encoded by the gene *fliC*. Based on its reaction with specific polyclonal antibodies and molecular weight, flagellin can be classified into two types: “a” type and “b” type. “A” type is a heterologous group of proteins with molecular weight ranging from 45 to 52 kDa; “b” type is a homologous protein with a molecular weight of 53 kDa (Brimer and Montie, 1998). Each stain of bacteria only expresses one type of flagellin and does not undergo antigenic variation (Winstanley et al., 1996). During infection, flagella can adhere to epithelial cells and the component flagellin can be recognized by Toll like receptor 5 (TLR5) to elicit a strong NF-κB-mediated inflammatory response (Miao et al., 2007). The presence of flagella in bacteria has been linked to acute infection in disease models (Brimer and Montie, 1998; Feldman et al., 1998), yet bacteria with down-regulation of flagella or aflagella are frequently found in chronic infections (Wolfgang et al., 2004).

Bacteria organize themselves to form a structured community on a surface, which is termed biofilm and intricately linked to QS (Bjarnsholt et al., 2009). Bacterial communities are embedded in extracellular polymeric substance (EPS), the major component of biofilm (50%-90% volume). EPS is comprised of polysaccharides, nucleic acids, proteins, and lipids. It confers physical and chemical resistance to mechanical forces and toxic chemical penetration, such as antibiotics and host defense molecules

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(Hall-Stoodley and Stoodley, 2009; Lieleg et al., 2011). The bacterial transcriptional profile in a biofilm is tailored to limit the consumption of oxygen and nutrition to slow growth rate. In the meantime, bacteria in a biofilm upregulate stress response genes, leading to increased antibiotic resistance (Mah and O'Toole, 2001). The initiation of biofilm is mediated by adhesions, such as type 4 pili, flagella, and fimbria (Mikkelsen et al., 2011). This process partly depends on the Las and Rhl QS systems and partly on environmental signals (Lopez et al., 2010). After the initiation, bacteria multiply as microcolonies and secrete EPS to form a matrix. The mature biofilm forms a mushroom-shaped structure. There are multiple channels in the structure to connect to outside environment for exchanges of waste and nutrient (Kaplan, 2010). Subsequently, the individual bacterium can shed off from the biofilm and be released to environment to begin a new cycle of biofilm formation.

A number of other virulence factors also contribute to the pathogenesis of *PA*. Lipopolysaccharide (LPS) localizes in the outer leaflet of *PA* membrane, consisting of membrane-anchored lipid A, a polysaccharide core region, and highly variable O-specific polysaccharide (O-antigen or O-polysaccharide) (King et al., 2009). LPS has been subjected to intensive study due to its strong antigenicity and other important characteristics. Proteases contribute to the pathogenesis by mediating the degradation of immunoglobulin and disruption of epithelial tight junctions (Kipnis et al., 2006). Alkaline protease is a zinc metalloprotease and able to degrade host complement proteins and fibronectin (Laarman et al., 2012). It is reported that alkaline protease is able to degrade free flagellin and hence prevents immune detection of flagellin by TLR5 (Bardoel et al., 2011). Elastase B (LasB) has been reported to degrade opsonizing lung

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surfactant proteins A and D (Mariencheck et al., 2003). The virulence of *PA* is attenuated with ΔLasB mutant (Kuang et al., 2011). Protease IV is a serine protease that can cause erosion of corneal epithelium through the degradation of complement proteins, immunoglobulins, and fibrinogen (Engel et al., 1998). In sum, *PA* possesses an arsenal of virulence factors involved in the pathogenesis of infection (Fig. 6).

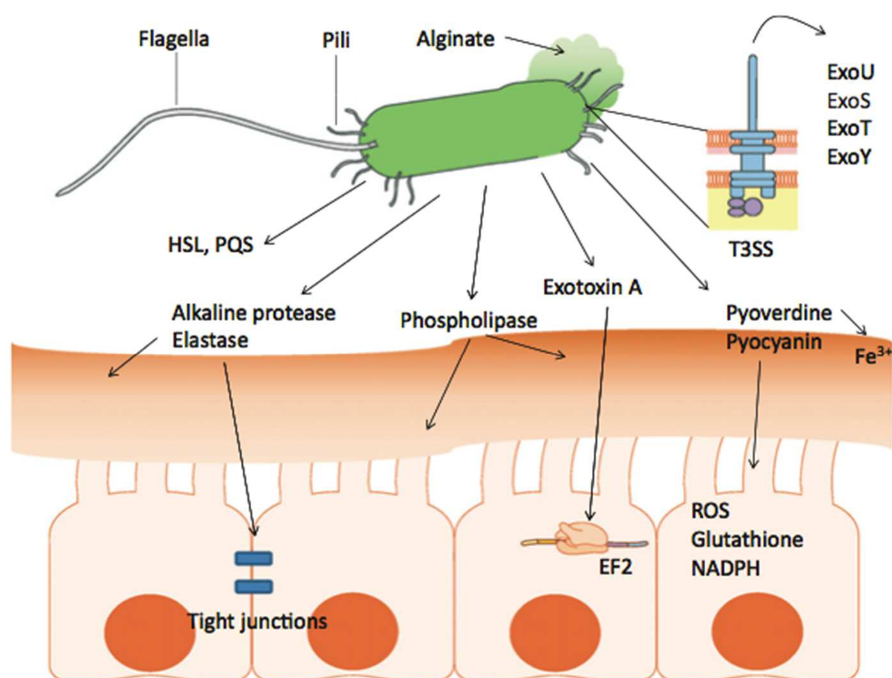

Figure 6 Virulence factors produced by *PA*. Flagellin and type 4 pilli are major adhesins to provide adhesion to epithelial cells. Flagellin and pilli, along with LPS, are also major inducers of inflammation. TTSS, once activated, is able to inject cytotoxins directly into host cells. Proteases can degrade host immune components and disrupt tight junctions to facilitate bacterial dissemination. Phospholipase can target host cell membranes. Pyocyanin can disrupt the redox cycling system in host cells. Pyoverdine captures Fe^{3+} to provide nutrition for bacteria. (Gellatly and Hancock, 2013)

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Animal models of *PA* keratitis

The devastating damage of infection is not only caused by virulence factors from bacteria, but also caused by host inflammatory response. The interplay between pathogens and the host demands live animal models to study ocular infection, aiming at new therapy development. The first animal model used in *PA* keratitis studies is New Zealand White rabbit. Hessburg and coworkers developed a *PA* keratitis model by passing a contaminated silk suture through rabbit corneal stroma (Penn et al., 1963). This model was used in earlier studies to explore the mechanisms of *PA* proteases (Wilson, 1970), and the effects of antibiotics on *PA* keratitis (Penn et al., 1963). Later, Kessler et al. developed an intrastromal injection model: *PA* was directly injected into the corneal stroma (Kessler et al., 1977). They used this model to study the proteolytic activity induced by heat-killed *PA* in rabbit corneas. They found a massive infiltration of polymorphonuclear leukocytes (PMNs) occurred during *PA* challenge; corneal damage could be due to the proteolytic enzymes produced by the host. The method of intrastromal inoculation has since been broadly used to study *PA* virulence factors and to develop novel therapies in rabbits. Subsequently, investigators developed a topical inoculation model. As has been mentioned previously, intact corneas are extremely resistant to *PA* infection. Therefore, corneal scratch (Blaylock et al., 1990; Frucht-Pery et al., 1995; Mannis, 2002), corneal abrasion (Michalova et al., 1996), or removal of corneal epithelia (Michalova et al., 1996) was used to break the barrier functions of the corneal epithelium before topical inoculation to induce *PA* keratitis in rabbits.

The advantage of using rabbit as an animal keratitis model is the large size of the cornea. Therefore, many parameters used in human keratitis can be used in rabbits

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(Johnson et al., 1990). For example, the presence of fibrin in anterior chamber in *PA* keratitis can be easily visualized in rabbits, but not in mice. However, rabbits are formidably expensive to use for research, and many reagents, such as anti-rabbit antibodies, are not commercially available for rabbit research. Mice, on the other hand, have a number of advantages. Mice are relatively inexpensive, and can be bred in large quantities in a short time period. Statistically significant number of mice can be used in each experiment. A multitude of reagents are commercially available for mouse studies. In addition, numerous strains, inbred or outbred, with different genetic background are available in use for different study purposes. Specifically, the availability of genetically modified mice allows researchers to study specific gene functions directly by knocking in or out certain genes. Therefore, mice are currently widely used in *PA* keratitis studies.

Gerke and Magliocco first reported to use mice as a *PA* keratitis model (Gerke and Magliocco, 1971). They successfully induced keratitis by making a 2-mm scratch in mouse cornea before topical inoculation of *PA*. They studied the corneal and ocular pathology of mouse keratitis and found that mice shared common characteristics with other animals as well as with humans. They also experimented different wounding methods: a 2-mm scratch, three needle incisions, a needle puncture, and direct intrastromal injection. All four methods of wounding followed by *PA* inoculation can induce a typical course of pathology in mouse cornea. They concluded that corneal wounding is necessary to induce infection, but the absolute extent and methods of making the wound are not critical. Yet, they found the intrastromal injection produced the most consistent pathology among individual mouse. Most importantly, they demonstrated that the severity of pathology is not quantitatively related to the number of

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PA in the cornea; the pathology lingers beyond the clearance of pathogens, further justifying the necessity of animal models to study host response to infection.

Both the corneal scratch and intrastromal injection models have proved of significant value in studying the immune response to *PA* keratitis. Although the intrastromal injection model provides more consistent pathology between mice, it bypasses the corneal epithelial layer to directly induce pathology in the stroma. The corneal scratch model is more clinically relevant, giving that *PA* keratitis usually occurs after corneal epithelial abrasion in patients. The corneal scratch model allows us to study innate immunity of corneal epithelial cells in response to infection. The variations in this procedure can be controlled by experienced hands. Therefore, we use the scratch model in this study to induce *PA* keratitis.

Innate immunity

Since we live in an environment full of various microorganisms, one of the fascinating problems in immunology is how host cells detect the presence of foreign intruders. In the early 1900's, the discovery of phagocytes by Elie Metchnikoff established the roots of innate immunity (Chang, 2009). Innate immunity, an immediate defensive system, detects constitutive and conserved molecules unique to microorganisms (Medzhitov, 2001). Some of the microbial gene products provide housekeeping functions and are essential to microbial survival, such as LPS and lipoteichoic acids (LTAs) on bacterial cell wall. Although different strains and species of microorganisms may have their unique chemical structures of housekeeping gene products, they are always found to have common molecular patterns highly conserved and invariant in a given class of microbes. For example, Gram-negative bacteria

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constitutively express LPS. The O-antigen portion of LPS is variable in different species of bacteria; however, the lipid-A portion remains invariant and is found in all Gram-negative bacteria. The conserved molecular patterns in microorganisms are named pathogen-associated molecular patterns (PAMPs). Accordingly, the receptors that recognize PAMPs in innate immune system are called pattern-recognition receptors (PRRs) (Janeway, 1989).

PRRs in vertebrate innate immune system include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs), Nod-like receptors (NLRs), and cytosolic DNA receptors including absence in melanoma 2 (AIM2) (Takeuchi and Akira, 2010). These PRRs are populated either on cellular membranes or in cytosolic compartments to detect danger signals. Furthermore, different families of PRRs can recognize the same ligand in different cellular locations. For example, flagellin can be recognized by membrane bound TLR5 and cytosolic NLR4 (Broz and Monack, 2013) . Such a system allows host cells to detect danger signals in multiple levels to ensure no danger signals escape surveillance.

Toll-like receptors

Although the innate immune system has been discovered for over a century, its importance was overshadowed by the fascinating adaptive immunity for a long period of time. The discovery and characterization of TLRs have incited keen interest in the field of innate immunity. Toll gene was first identified in *Drosophila* as a maternal-effect gene that functions in fruit fly embryo development (Hashimoto et al., 1988). Later, Toll mutant was found to be defective against fungal infection, demonstrating its function in the immunity of fruit flies (Lemaitre et al., 1996). Subsequently, mammalian homologues

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of Toll were identified (Anderson, 2000; Medzhitov et al., 1997), and the importance of innate immunity has since been greatly appreciated.

TLRs belong to the type I transmembrane receptor family. They consist of three components: an amino (N)-terminal extracellular leucine-rich repeat (LRR) domain, a single transmembrane region, and a carboxyl (C)-terminal intracellular Toll/ IL-1 receptor (TIR) domain (Gay and Gangloff, 2007). Thus far, ten TLRs (TLR 1-10) have been identified in human and thirteen TLRs (TLR1-13) in mice (De Nardo, 2015). TLRs can either localize on host cell plasma membrane to recognize microbial surface molecules (e.g. TLR5 and TLR2), or in endolysosomal compartment to recognize microbial nucleic acids (e.g. TLR3). Table 1 summarizes the cellular localizations and microbial ligands of TLRs. In most cases, ligand binding induces dimerization of TLRs (Bell et al., 2005; Gay et al., 2006); however, the endosomal TLRs, including TLR7, TLR8, and TLR9, are reported to preform dimers before ligand recognition (Latz et al., 2007; Tanji et al., 2013). The dimerization broadens the array of ligands recognized by TLRs. For examples, TLR2 can form dimers with either TLR1 or TLR6 to recognize triacylated lipoproteins or diacylated lipoproteins, respectively (Takeuchi et al., 2001; Takeuchi et al., 2002).

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Table 1 TLR expression, localization, and ligands. Slight modification. (De Nardo, 2015)

TLR	Species	Localisation	Microbial ligands
TLR1	Human and mouse	Plasma membrane	Triacyl lipoproteins
TLR2	Human and mouse	Plasma membrane	Lipoproteins, zymosan, mannan, peptidoglycan, lipoteichoic acid,
TLR3	Human and mouse	Endolysosomal membrane	Viral dsRNA
TLR4	Human and mouse	Plasma and endolysosomal membrane	LPS
TLR5	Human and mouse	Plasma membrane	Flagellin
TLR6	Human and mouse	Plasma membrane	Diacyl lipoproteins, lipotechoic acid, zymosan
TLR7	Human and mouse	Endolysosomal membrane	Viral and bacterial ssRNA
TLR8	Human and mouse	Endolysosomal membrane	Viral and bacterial ssRNA
TLR9	Human and mouse	Endolysosomal membrane	Viral and bacterial CpG DNA, DNA:RNA hybrids
TLR10	Human	Plasma membrane	Unknown
TLR11	Mouse	Endolysosomal membrane	Profilin and flagellin
TLR12	Mouse	Endolysosomal membrane	Profilin
TLR13	Mouse	Endolysosomal membrane	Bacterial 23S ribosomal RNA (rRNA)

TLR4 is the first discovered mammalian Toll. It recognizes LPS on Gram-negative bacteria (Hoshino et al., 1999; Medzhitov et al., 1997; Poltorak et al., 1998; Qureshi et al., 1999). TLR4 is expressed on a variety of cells types, predominantly on immune cells such as macrophages and neutrophils (Medzhitov et al., 1997). Massive inflammation caused by TLR4 signaling transduction has been implicated in fatal sepsis syndrome (Karima et al., 1999). TLR5 recognizes flagellin, the only protein PAMP sensed by TLRs (Hayashi et al., 2001). Flagellin is the main structural protein of flagellum, a component vital for bacterial mobility. TLR5 is only expressed in the basolateral side of simple epithelium and the basal layer of stratified epithelia (Gewirtz et al., 2001). It is believed that the polarized expression of TLR5 is essential for the host

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to distinguish pathogenic and commensal pathogens, since pathogenic organisms can cross the epithelial barrier (Medzhitov, 2001).

Broadly speaking, TLR activation triggers two major signaling pathways: MyD88-dependent and TRIF-dependent pathways (Fig. 7). Both MyD88 and TRIF contain TIR domain that can bind to the TIR domain of TLRs to connect the receptors to downstream signaling molecules (O'Neill and Bowie, 2007). All TLRs, except TLR3, recruit MyD88 to initiate signaling transduction. Due to suboptimal electrostatic surface charges, TLR2 and TLR4 require additional bridging/sorting adaptor, MAL (also known as TIRAP), to couple to the TIR domain of MyD88 (Dunne et al., 2003; Fitzgerald et al., 2001; Horng et al., 2002). After binding to TLRs, MyD88 recruits the IL-1 receptor associated kinases (IRAKs) through homotypic protein-protein interaction on the shared death domain (DD) (Wesche et al., 1997). Activation of IRAKs recruits the E3 ubiquitin (Ub) ligase, TNFR-associated factor 6 (TRAF6), to the complex. Once activated, TRAF6 is released into the cytosol and forms a complex with TAK1, TAB1 and TAB2/3 (Qian et al., 2001). The newly formed cytosol complex activates an IKK complex to mediate phosphorylation and degradation of IκB. Without the inhibition of IκB, NF-κB is released and translocated into the nucleus to initiate inflammatory cytokine gene transcription (Napetschnig and Wu, 2013).

TLR3 recruits and can directly interact with TRIF to initiate TRIF-dependent signaling pathway (Oshiumi et al., 2003; Yamamoto et al., 2003a; Yamamoto et al., 2002). While activation of TLR4 in the plasma membrane triggers MyD88 signaling pathway, once TLR4 is endocytosed into endosomal compartments, it can recruit TRIF through the bridging adaptor TRAM to initiate TRIF-dependent signaling pathway

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(Kagan et al., 2008; Yamamoto et al., 2003b). Engagement of TRIF with TLR3 or TLR4 provides docking sites for both TRAF6 and TRAF3 via TRAF6 binding motifs. TRAF6 recruitment activates RIP1 kinase to mediate NF-κB signaling cascade. TRAF3 can associate with TBK-1 to activate IRF3, and eventually induce the type I interferon production (Gohda et al., 2004; Sato et al., 2003).

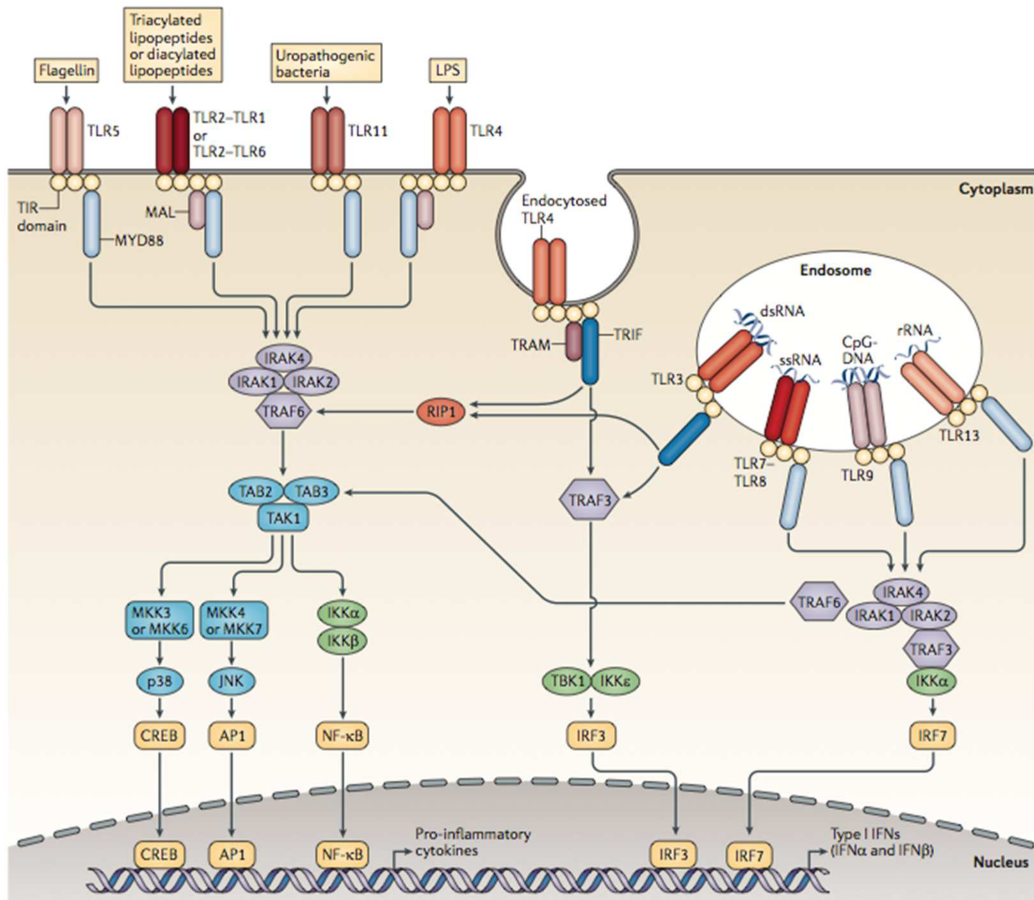

Figure 7 Toll-like receptor signaling pathways. TLR signaling is mainly mediated by MyD88-dependent or TRIF-dependent pathway to induce the expression of pro-inflammatory cytokines and type I interferons. (O'Neill et al., 2013)

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Activation of TLRs initiates a cascade of signal transduction events to promote the expression of pro-inflammatory cytokines and chemokines (Chen et al., 2005; Kumar et al., 2006; Vora et al., 2004). TLR stimulation also mediates the expression of antimicrobial peptides, such as defensins, to kill pathogens directly (Redfern et al., 2011). Chemokines recruits inflammatory cells from limbal capillaries to the site of infection. Neutrophils are the predominant cell type recruited and essential for bacterial killing and wounding healing. The generation of reactive oxygen species (ROS) (Leal et al., 2012) and the formation of neutrophil extracellular traps (Brinkmann et al., 2004) are believed to play critical roles in neutrophil-mediated bacterial killing. Inflammatory cytokines also play an important role in microbial keratitis. IL-1β is a major mediator of inflammatory response. It is an early responsive cytokine upon microbial keratitis, and is able to induce other mediator expression and neutrophil infiltration. Depletion or deficiency of IL-1β renders mice unable to clear pathogens (Karmakar et al., 2012; Rudner et al., 2000). A certain amount of inflammatory response is beneficial to pathogen clearance; however, prolonged unrestrained inflammation is devastating and contributes to corneal destruction. The production of matrix metalloproteinase (MMPs) from neutrophils can degrade base membrane and disrupt normal collagen matrix in the stroma, resulting in corneal damage or even perforation (Nathan, 2006). A reduction of neutrophil recruitment is related to reduced corneal damage in *PA* keratitis (Hazlett et al., 1992). Therefore, the TLR-mediated inflammatory response must be precisely regulated.

Flagellin-mediated innate immune protection

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The concept of endotoxin tolerance was first described by Beeson in 1946 (Beeson, 1946). Endotoxin tolerance refers to: pre-exposure of innate immune cells (*in vitro*), or hosts (*in vivo*), with a low dose of endotoxin (e.g. LPS), renders them transiently unresponsive to a subsequent large dose of endotoxin. For a long period of time, the mechanism of endotoxin tolerance remained elusive. Later, researchers found that alterations of the LPS-TLR4 signaling pathway are responsible for the tolerance (Fan and Cook, 2004; Medvedev et al., 2000; Nomura et al., 2000). Endotoxin tolerance is not only due to suppressing inflammatory signaling mediators, but also increasing anti-inflammatory signaling molecules (Biswas and Lopez-Collazo, 2009) (Fig. 8). Subsequently, investigators found that other microbacterial products, such as LTAs, CpG DNA, and flagellin, can also induce protective innate immunity (Lehner et al., 2001; Mizel and Snipes, 2002; Yeo et al., 2003). Besides self-tolerance, most microbial products can desensitize the host immune response to a different type of microbial products, a phenomenon termed cross-tolerance. Lehner MD et al. reported that pre-stimulation of both immune cells and mice with highly purified LTA resulted in the suppression of signal transduction and cytokine release on subsequent LPS challenge, and vice versa (Lehner et al., 2001). The Yi AK group also demonstrated that pre-stimulation of RAW264.7 cells with CpG DNA induced self- and cross-tolerance to a second challenge with CpG DNA and LPS, respectively (Yeo et al., 2003).

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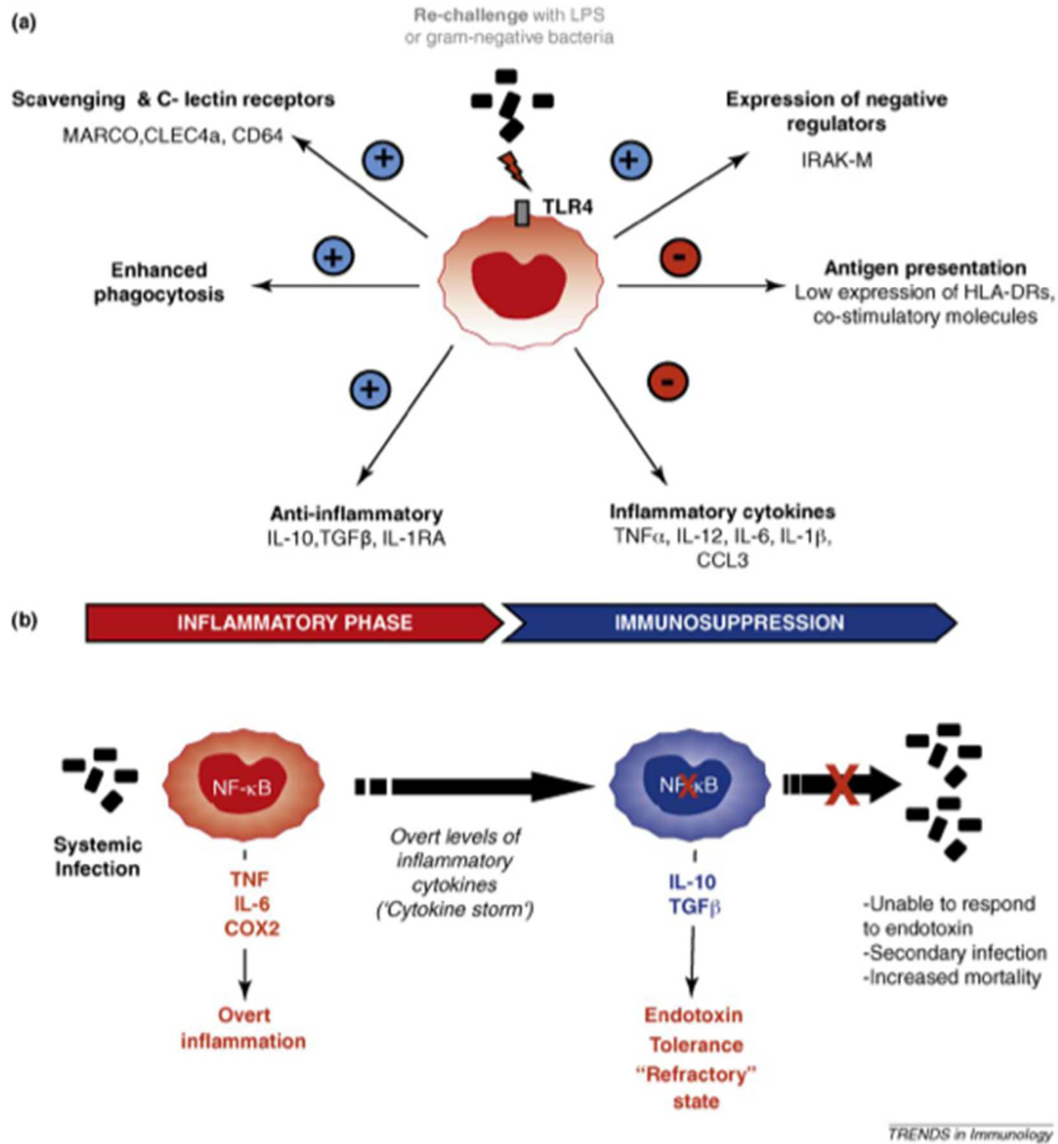

Figure 8 Endotoxin tolerance. (A) When re-challenged with LPS, host cells show down-regulation of inflammatory cytokines and compromised antigen presentation ability, yet up-regulation of anti-inflammatory cytokines, negative regulators, scavenging receptors, and enhanced phagocytosis. (B) Endotoxin tolerance suppresses the key inflammatory regulatory factor NF-κB to turn host response into a refractory state. (Biswas and Lopez-Collazo, 2009)

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Mizel and coworkers first reported that prior exposure of human monocytes with a minute amount of flagellin resulted in a state of hypo-responsiveness to a second large dose of flagellin (Mizel and Snipes, 2002). They demonstrated that flagellin-induced innate protection was not due to any change in the expression levels of TLR5 or IRAK, but was associated with blocking the release of IRAK from TLR5, rendering IRAK without kinase activity. Besides immune cells, Sun and colleagues proved that flagellin-induced tolerance also occurred in polarized intestinal epithelial cells (Sun et al., 2007). Our previous data show that flagellin can induce a tolerant response in human corneal epithelial cells (HCECs) (Kumar et al., 2007). To date, flagellin has been found to induce protection against infection caused by a variety of pathogens.

Flagellin-induced protection is mediated by the activation of innate immunity through TLR5 or NLR4 signaling pathway, independent of adaptive immunity (Liu et al., 2014; Zhang et al., 2014a). Flagellin pretreatment protects mice from pneumonia caused by *PA* or *Streptococcus pneumoniae* (Munoz et al., 2010; Tolle et al., 2015; Yu et al., 2010). Flagellin is also able to protect mice from intestinal rotavirus infection (Zhang et al., 2014a). Our lab demonstrates that Flagellin pretreatment protects mouse cornea from bacterial keratitis (*P. aeruginosa*) (Kumar et al., 2010; Kumar et al., 2008; Yoon et al., 2013) or fungal keratitis (*Candida albicans*) (Gao et al., 2011; Liu et al., 2014). Flagellin pretreatment alleviates clinical symptoms, enhances bacterial clearance, and decreases neutrophil infiltration and pro-inflammatory cytokine secretion in infected corneas. At the same time, flagellin pretreatment increases the expression of cathelicidin-related antimicrobial peptide (CRAMP), inducible nitric oxide synthase (iNOS), and C-X-C motif chemokine 10 (CXCL10). Flagellin can directly induce the

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expression of antimicrobial peptides in epithelial cells, or indirectly through dendritic cells, which induce protective gene expression to mediate the protective effect. These data demonstrate that consistent with endotoxin tolerance, flagellin-induced protection is not only associated with reduced expression of pro-inflammatory cytokines, but also related to enhanced expression of anti-inflammatory cytokines and antimicrobial molecules to counter the inflammatory response and simultaneously enhance the innate defense against infection.

In order to get a global picture of the altered gene expression in flagellin-induced protection, we pretreated mouse corneas with flagellin before the inoculation of *PA* stain *PAO1*, and collected mouse corneal epithelial cells at 6 hpi to run a genome-wide cDNA microarray (Gao et al., 2013). As shown in Fig. 9, comparing with naïve corneal epithelial cells, *PAO1* infection resulted in a total of 675 genes with altered expression, with 497 up- and 178 down-regulated genes; flagellin pretreatment before *PAO1* infection resulted in a total of 929 genes with altered expression, with 890 upregulated genes. While comparing the gene expression profiles in *PAO1* infected corneal epithelial cells with flagellin pretreatment to without flagellin pretreatment, we found that there were a total of 209 differentially expressed genes, with 157 up- and 52 down-regulated genes. The differentially expressed genes provide insights into their functions in flagellin-induced protection. For example, the suppressed genes after flagellin pretreatment (e.g. IL-1β and MMP13) suggest that they may be related to inflammatory response and hence may cause damage to the cornea if left uncontrolled. The augmented genes (e.g. S100A8/A9), on the other hand, may possess counter inflammatory function or antimicrobial activity. Based on the cDNA microarray data, we

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constructed an innate defense network in flagellin-induced tolerance against microbial infection in corneal epithelial cells, as shown in Fig 10 (Gao et al., 2013).

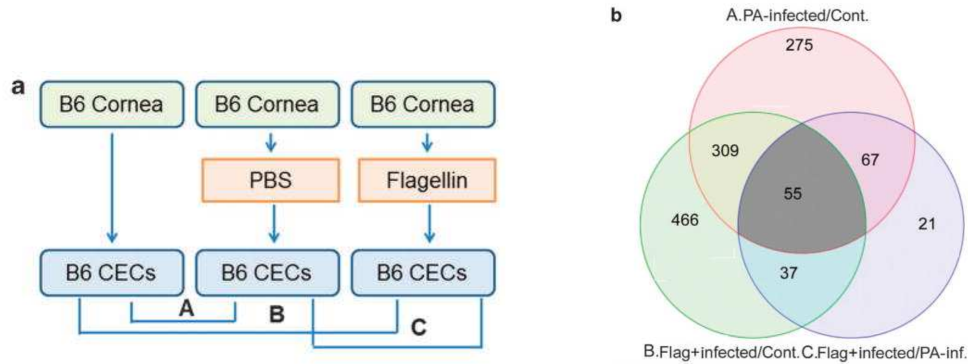

Figure 9 Gene expression profiles of mouse corneal epithelial cells after *PA* strain *PAO1* infection with or without flagellin pretreatment. (A) Experimental design. Mouse corneal epithelial cells were pretreated with flagellin for 24h before *PAO1* challenge. Samples were collected at 6 hpi for cDNA microarray analysis with naïve corneal epithelial cells as control. (B) *PAO1* infection with and without flagellin pretreatment altered the expression of 929 and 675 genes, respectively; there are a total of 209 differentially expressed genes in corneal epithelial cells when compare *PAO1* infection with flagellin pretreatment to *PAO1* infection without flagellin pretreatment. (Gao et al., 2013)

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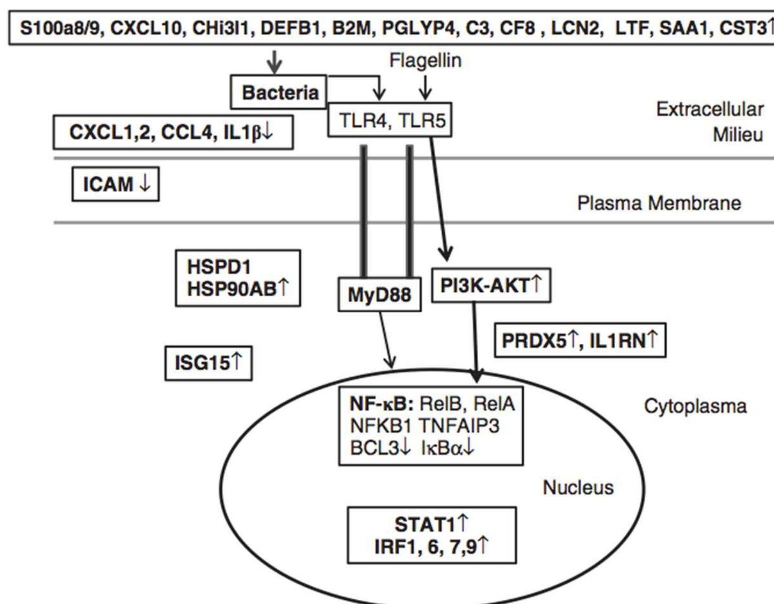

Figure 10 A network of flagellin-induced protection in corneal epithelial cells. Flagellin pretreatment may further induce, have no effect, or suppress gene expression after subsequent microbial challenge, forming a complex orchestrated counter inflammatory network. The altered gene products may be located in the cytoplasm, nucleus, membrane, or secreted in extracellular space. (Gao et al., 2013)

Flagellin has been explored to use as a vaccine candidate or a vaccine adjuvant to protect mouse from an array of microbial pathogen. Harjai et al. reported immunization of mice with divalent flagellin, including type “a” and “b” provides homologous and heterologous protection against experimental *PA* urinary tract infection (Sabharwal et al., 2016). The adjuvant effect of flagellin has been reported in many vaccines to induce a potent and broad-spectrum immune protective effect against various types of infection, including *Yersinia pestis* (Honko et al., 2006), *Mycobacterium tuberculosis* (Le Moigne et al., 2008), *influenza viruses* (Hong et al., 2012; Skountzou et al., 2010), *West Nile viruses* (McDonald et al., 2007), and *human immunodeficiency*

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virus (Vassilieva et al., 2011). Flagellin was fused to a vaccine gene to generate a DNA vaccine to induce protective immunity against lethal *PA* pneumonia (Saha et al., 2007). The advantages of flagellin as an adjuvant are: (1) Flagellin can activate host immune system through the interaction with plasma membrane surface receptor TLR5 and cytosolic receptor NLR4; (2) Flagellin is a stable protein that can be fused to other vaccine proteins to incorporate multiple antigens in one molecule (Cuadros et al., 2004); (3) Less antigen and adjuvant might be needed due to the adjuvant effect of flagellin (Cuadros et al., 2004; Huleatt et al., 2007).

The flagellin protective effect has been extensively investigated to other areas beyond infection. A pharmacologically optimized flagellin derivative, CBLB502, was developed to study its effects. It is reported that Flagellin is a potent radioprotectant to protect tissues from radiation-induced injury, indicating flagellin can be used as a mean of supportive care to radiotherapy (Burdelya et al., 2012; Burdelya et al., 2008). Administration of flagellin before induction of ischemia or immediately after reperfusion can protect mice from ischemic-reperfusion syndrome (Fukuzawa et al., 2011). In addition, flagellin can inhibit the growth of TLR5-expressiving tumors, such as lung adenocarcinoma A549 (Burdelya et al., 2013; Burdelya et al., 2012). Furthermore, administration of CBLB502 targets the liver to induce the expression of immunomodulatory factors and to recruit immune cell infiltration, resulting in growth inhibition of liver metastases from tumors of various origins, regardless of their TLR5 expression status (Burdelya et al., 2013). Therefore, the protective effects of flagellin are broad.

The IL-10 family

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As previous stated, both causative organisms and host immune system contribute to tissue damage. Therefore, the inflammatory response has to be finely tuned and controlled in a timely manner. Several mechanisms have evolved to balance the inflammatory response. One of them is the expression of cytokines that possess anti-inflammatory and immunomodulatory characteristics, such as IL-10. IL-10 was originally reported as a cytokine to counter IFN-γ response by T lymphocytes (Liu et al., 1994). Ten years after the discovery of IL-10, various genetic approaches have identified several cytokines structurally and functionally related to IL-10, which are collectively referred to as the IL-10 family. Based primarily on their biological function, the IL-10 family can be further grouped into three subgroups. The first group includes only IL-10 itself. The second group is also referred to as the IL-20 subfamily, including IL-19, IL-20, IL-22, IL-24 and IL-26. The third group is the type III IFN group, containing IL-28A, IL-28B, and IL-29 (Ouyang et al., 2011).

IL-24 was the first discovered homolog to IL-10. It was identified by subtraction hybridization from a human melanoma cell line (Jiang et al., 1995). IL-24 was initially named melanoma differentiation associated gene-7 (MDA-7). MDA-7 protein expression is found in normal melanocytes, gradually decreases during the progression of melanomas, and disappears in metastatic melanomas (Ekmekcioglu et al., 2001; Huang et al., 2001). Its cytokine property was not originally reported due to the abnormal long signaling peptide encoded by MDA-7 gene. The IL-19 and IL-20 genes were discovered by sequence database search for IL-10 homologs (Blumberg et al., 2001; Gallagher et al., 2000). IL-22 was identified by cDNA subtractive hybridization as a gene expressed in a mouse T cell line stimulated with IL-9, and was originally named IL-10-related T

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cell-derived inducible factor (IL-TIF) (Dumoutier et al., 2000a). IL-26 was discovered as a cytokine expressed in *Herpesvirus saimiri* (HVS)-transformed human T cells, and originally designated as AK155 (Knappe et al., 2000). Thus far, IL-26 has been reported in humans and other vertebrates, but is absent in mice. IL-28A, IL-28B, and IL29 were also identified by sequence database search for novel genes similar to IFN and the IL-10 family cytokines (Kotenko et al., 2003; Sheppard et al., 2003). They were later grouped into type III IFNs due to their functional similarity to type I IFNs (Fox et al., 2009).

The genes encoding the IL-10 family cytokines are mapped to two chromosomal loci, chromosome 1q32+2 and chromosome 12q14+3 (Kotenko, 2002) (Figure 11). The genes for IL-10, IL-19, IL-20, and IL-24 cluster at chromosome 1q32+2, with IL-19, IL-20, and IL-24 genes positioned in a head to tail manner and IL-10 gene in the opposite direction toward the telomere. The genes for IL-22 and IL-26 are localized in chromosome 12q14+3 within 100kb of the IFNG gene, orienting toward the telomere. The IL-10 family cytokines share similar gene structures (Fig. 12). There are 5 exons (exons 1-5) in the coding region and conserved positions of the intron/exon junctions of each of the genes (Dumoutier et al., 2000b; Kotenko, 2002). However, IL-19, IL-22, and IL-24 genes have two additional axons (exons 1a and 1b) upstream of the first common exon 1 (Dumoutier et al., 2000b; Gallagher et al., 2000; Huang et al., 2001). In addition, IL-19 gene has at least two alternative exons 1a, exon1a1 and exon1a2 (Gallagher et al., 2000). The exon 1a2 in IL-19 gene and exon 1a in the IL-24 gene encode long abnormal signal peptides, the presence of which in IL-19 and IL-24 proteins limits their secretory ability. Alternatively, when exon 1a1 is present instead of exon 1a2 in IL-19

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mRNA or exon 1a is spliced out in IL-24 mRNA, these mRNAs are translated into proteins with canonical signal peptides. The IL-19 and IL-24 proteins with the shorter canonical signal peptides can be secreted into extracellular space at higher level. While the proteins of IL-10 and IL-26 are biologically active as intercalated dimers (Fickenscher and Pirzer, 2004; Zdanov et al., 1995), IL-19, IL-20, IL-22, and IL-24 are functionally active as monomers (Chang et al., 2003; Nagem et al., 2002; Pletnev et al., 2003; Sauane et al., 2003).

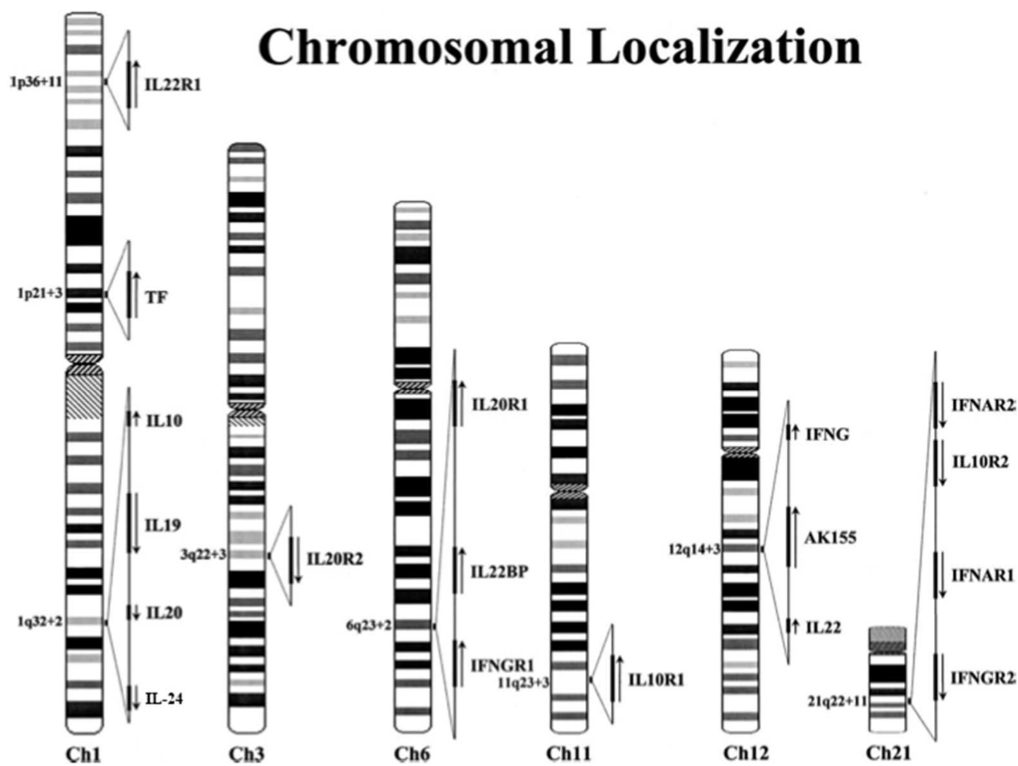

Figure 11 Chromosomal localization of the genes encoding cytokines from the IL-10 family and receptors for the IL-10 family. (Kotenko, 2002)

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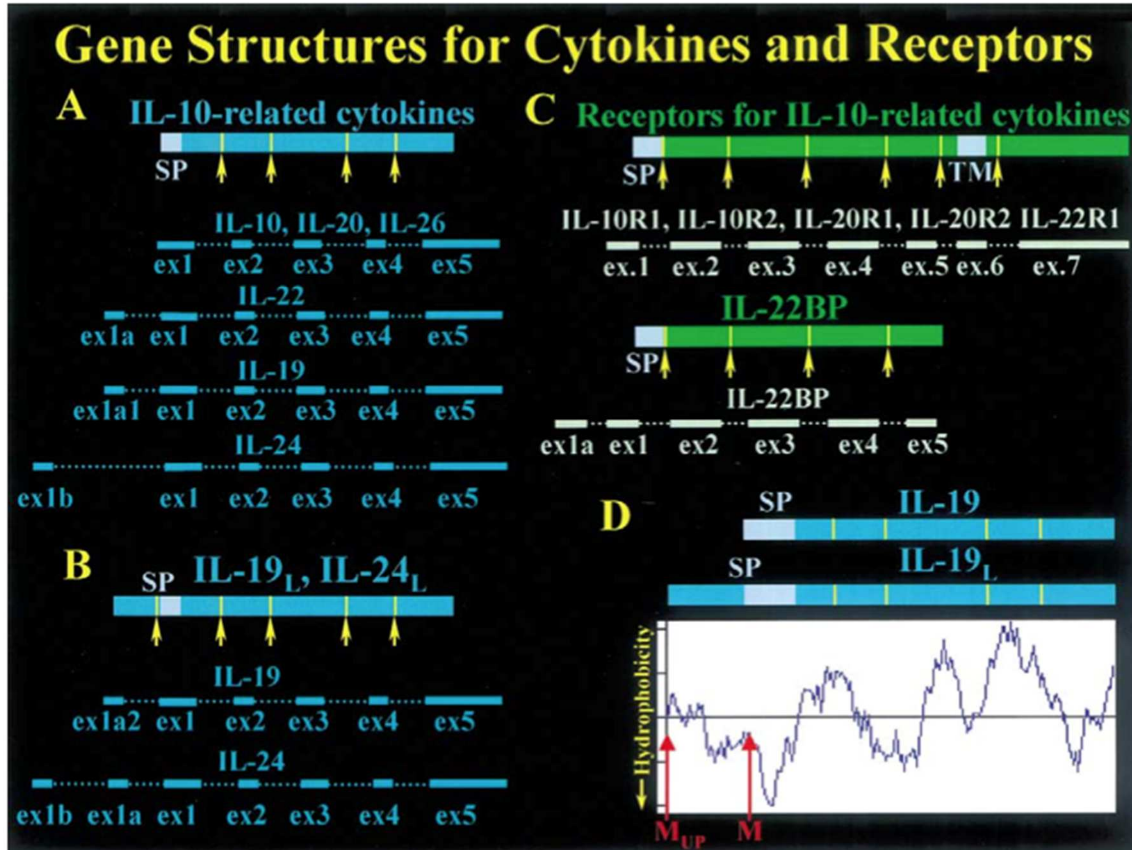

Figure 12 Genomic structures of IL-10 family cytokines and their receptors. Proteins are depicted as thick colored bars. Arrows indicate former intron positions. Gene transcripts are shown as strings of lines (exons) with dotted lines (introns). (A and B) There are five exons in the coding region of the genes of IL-10 family cytokines. IL-19 and IL-22 transcripts have one additional exon (ex1a), whereas IL-24 transcripts have two additional exons (ex1a and ex1b). IL-19 has two alternative transcript variants of ex1a (ex1a1 and ex1a2). When ex1a2 is present in IL-19 and ex1a is present in IL-24, an additional Met codon (M_{up}) precedes the Met codon (M) encoded by ex1. When translation starts from M_{up}, IL-19 and IL-24 proteins have elongated abnormal signal peptides. Otherwise, when translation starts from M, IL-19 and IL-24 proteins possess canonical signal peptides. (C) There are seven exons in the receptor genes for the IL-10 family cytokines, except IL-22BP gene, which has five exons. (D) Hydropathy plot of IL-19 protein. Arrows indicate positions of M_{up} and M codons. SP: signal peptide; TM: transmembrane domain. (Kotenko, 2002)

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Receptor complexes

Cytokines bind to cell surface receptors to trigger intracellular signal transduction to regulate downstream gene expression. Based on the primary structure of their extracellular domains, cytokine receptors can be classified into two groups: class I and class II receptors (Bazan, 1990a; Bazan, 1990b). The receptors for IL-10-related cytokines belong to the class II receptors (Ho et al., 1993). The extracellular domains of Class II receptors are characterized by the presence of two highly conserved disulfide bridges and two fibronectin type III sub-domains in tandem with specific pattern of proline and cysteine residues (Haque and Sharma, 2006; Kotenko and Langer, 2004), and the absence of intact "WSXWS" motif in the region close to cell membrane (Bazan, 1990b). The receptor binding of the IL-10 family cytokines follows the paradigm of the class II cytokine receptors, which was established based on the receptor complexes for IFN-γ and IL-10 (Bach et al., 1997; Kotenko et al., 1997; Kotenko and Pestka, 2000; Pestka et al., 1997). All the members of the IL-10 family signal through receptor complexes comprising of a long-chain type 1 receptor and a short-chain type 2 receptor (Trivella et al., 2010) (Table 2). For example, IL-10 signals through IL-10R1 and IL-10R2 receptor complexes. Moreover, sharing receptor components has been found to be a common feature in the IL-10 family. IL-10, IL-22, and IL-26 share IL-10R2 as the common receptor chain, while utilizing IL-10R1, IL-22R1, and IL-20R1 as a unique ligand binding chain, respectively (Kotenko et al., 1997; Xie et al., 2000). IL-19, IL-20, and IL-24 share the IL-20R1 and IL-20R2 heterodimer receptor complex. In addition, IL-20 and IL-24 can signal through the IL-22R1 and IL-20R1 receptor complex (Dumoutier et al., 2001). Specificity is attained by unique receptor-cytokine interaction so the

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interface is affinity tuned to transmit distinct signaling through shared receptor complex (Logsdon et al., 2012). The receptor sharing does not seem to have mutual competitive inhibition in their biological functions (Wolk et al., 2005). Additionally, IL-22 can bind to IL-22 binding protein (IL-22BP), a soluble type 1 receptor that can block IL-22 signaling.

Table 2 The IL-10 family cytokines and their transmembrane receptors. (Trivella et al., 2010)

IL-10 member	Type 1 receptor	Type 2 receptor
IL-10	IL-10R1	IL-10R2
IL-19	IL-20R1	IL-20R2
IL-20	IL-20R1	IL-20R2
	IL-22R1	IL-10R2
IL-22	IL-22R1	IL-10R2
	IL22BP	
IL-24	IL-20R1	IL-20R2
	IL-22R1	IL-20R2
IL-26	IL-20R1	IL-10R2
IFN-λs	INF-λR1	IL-10R2

The genes encoding the receptors for the IL-10 family cytokines are scattered in different chromosomes: IL-10R1 on chromosome 11, IL-10R2 on chromosome 21, IL-20R1 and IL-22 BP on chromosome 6, IL-22R1 on chromosome 1, and IL-20R2 on chromosome 3 (Fig. 11) (Kotenko, 2002). These genes also share conserved structures. The coding regions of each receptor gene contain 7 exons except the IL-22BP gene,

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which has 5 exons and encodes a soluble receptor (Fig. 12). Exon 1 encodes the 5'-UTR and the signal peptide; exon 2 to 5 encode the extracellular domain; exon 6 encodes part of the extracellular domain, transmembrane domain, and part of the intracellular domain; exon 7 encodes the rest of intracellular domain and the 3'-UTR (Mah and O'Toole, 2001).

In most cases, the extracellular domain of R1 receptor exhibits high affinity to ligands and is the ligand-binding chain in the receptor complex. However, it is reported that IL-24 exhibits significant binding to the R2 receptor, IL-20R2. The presence of R1 receptor, IL-20R1 or IL-22R1, dramatically increases binding affinity (Wang et al., 2002). Neither IL-20R1 nor IL-22R1 can bind IL-24 alone (Wang et al., 2002). The cytoplasmic domain of the receptors usually forms short α-helices organized in boxes, which are responsible for molecular interactions with Janus kinases (JAKs). R1 receptor has a long intracellular domain and it can be phosphorylated by JAK on Tyr residues after ligand-receptor engagement. R2 receptor has a short intracellular domain that is associated with JAK or Tyk2 tyrosine kinase, bringing an additional tyrosine kinase to the receptor complex (Bach et al., 1996; Kotenko et al., 1995; Kotenko et al., 1997). Ligand binding triggers conformational change of the receptor complex, and cross-activation of JAKs (Kotenko et al., 1996). Subsequently, the long R1 intracellular domain recruits signal transducer and activator of transcription (STAT), which contain SH2 domains, to the receptor complex (Kotenko et al., 1999). STAT3 is the key transcription factor utilized by IL-10 and IL-20 subfamily cytokines, although STAT1 and STAT5 can also be activated by IL-10 and IL-22 (Kotenko et al., 2003). Eventually, STATs are phosphorylated by JAKs and then translocated as homo- or heterodimers

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into the nucleus to regulate target gene expression (Langer et al., 2004; Pestka et al., 2004) (Fig. 13).

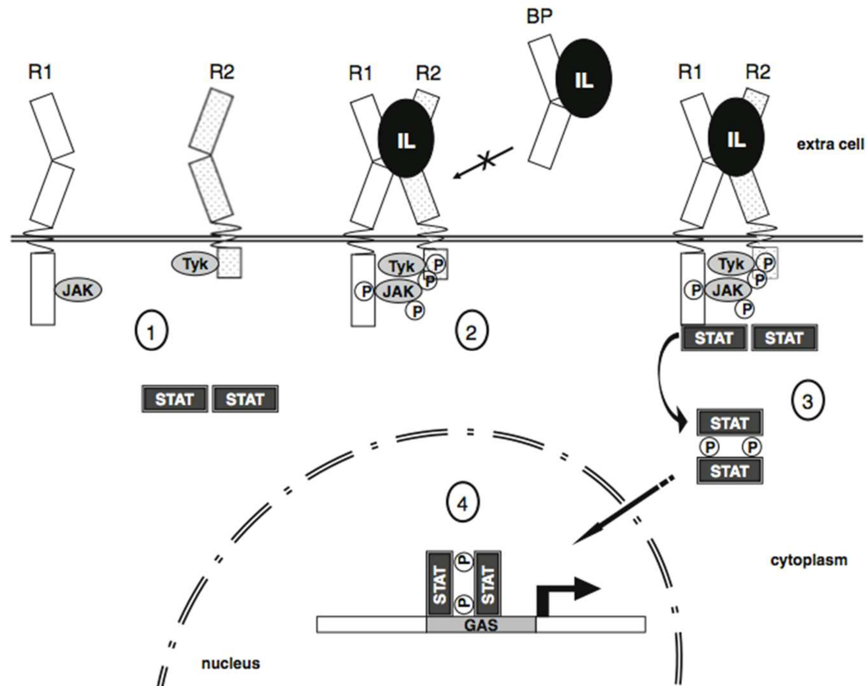

Figure 13 The schematic signaling pathway of the IL-10 family cytokines, based on the IL-22 set of receptors. (1) In static state, inactivated R1 and R2 chains are bound to JAKs. Inactivated STAT dimers are localized in the cytoplasm. (2) Ligand binding to the receptors induces phosphorylation of JAKs and the receptors. IL-22BP is a soluble decoy receptor that can bind to IL-22 to prevent signal transduction. (3) STATs are recruited to the receptor complex and get phosphorylated. (4) Activated STATs form dimers and translocate to the nucleus to regulated related gene expression. (Trivella et al., 2010)

JAK/STAT signaling pathway

In mammals, the JAK family consists of four members, JAK1, JAK2, JAK3, and TYK2 (Bromberg et al., 1999; Williams, 2000). The JAK proteins are large kinases and have a molecular weight of 120-140 kDa. They have seven homologous domains, named as Janus homology domain (JH) 1-7. JH1 is a functional catalytic kinase

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domain, phosphorylation of which would lead to JAK protein conformational changes to facilitate the interaction with its substrates. JH2 is a pseudokinase domain, which lacks enzymatic activity and may act as a regulatory site or a docking site for STATs. The JH3 and JH4 domains are homologous to SH2 domains. JH4-JH7 are localized in the amino terminal end of JAKs and involved in interaction with cytokine receptors and/or other kinases (Kisseleva et al., 2002). Phosphorylation of JAKs requires two JAKs from the same or different class to be proximal to each other. Different cytokine receptors may preferentially activate specific JAKs to transduce signals inside the cells.

Seven STATs have been identified in mammalian cells: STAT1-4, STAT5a, STAT5b, and STAT6 (Leonard and O'Shea, 1998). Their molecular masses range from 75 to 95 kDa. The STAT proteins share conserved structures, a SH2 domain and a C terminal tyrosine residue (Y705 in mice). The interaction of the SH2 domain with phosphorylated cytokine receptors may contribute to specific cytokine responses (Schindler, 1999). The tyrosine residue can be phosphorylated by JAKs, which would lead to STAT dimerization and subsequently translocation into the nucleus. STATs form homodimers or heterodimers through the interactions of the SH2 domain-phosphotyrosine of two monomers to form a reciprocal conformation (Grandis et al., 2000; Leung et al., 1995). The serine at position 727 (S727) in STAT3 can also be phosphorylated to enhance transcriptional activity (Boulton et al., 1995; Shen et al., 2004; Wen and Darnell, 1997; Wen et al., 1995). STATs bind to DNA through a central DNA-binding region to regulate related gene expression. After modulating gene expression, phosphorylated STATs can be dephosphorylated by tyrosine phosphatases and ready to for subsequent stimulation (Chung et al., 1997).

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STAT3 was first identified as a downstream signaling mediator of IL-6 two decades ago (Lutticken et al., 1994; Standke et al., 1994; Zhong et al., 1994). Besides IL-6, many other cytokines and growth factors can also activate STAT3, including cytokines using receptor chain gp130, IL-20R cytokines, granulocyte colony-stimulating factor (G-CSF), and growth factors that signal through protein tyrosine kinase receptors, etc. (Ruff-Jamison et al., 1994; Standke et al., 1994; Tian et al., 1994; Zhong et al., 1994). Increasing evidences prove that STAT3 plays important roles in immunity. Hyper immunoglobulin E syndrome (HIES) is a human immunodeficiency syndrome caused by autosomal dominant STAT3 inactivating mutations. Characteristic features in these patients include recurring bacterial or fungal infection, elevated pro-inflammatory response, and increased immunoglobulin E (IgE) in circulation (Freeman et al., 2009; Freeman and Holland, 2008; Minegishi, 2009; Minegishi and Karasuyama, 2009). On the other hand, over activation of STAT3 (e.g. single nucleotide polymorphisms) has been linked to human autoimmunity diseases, for example, psoriasis and multiple sclerosis (Jakkula et al., 2010; Tsoi et al., 2012). It is reported that some individuals with juvenile-onset autoimmunity and lymphoproliferation are due to de novo activating point mutations in STAT3 (i.e., GOF). GOF mutations in STAT3 elevate basal and cytokine-induced STAT3 transcriptional activity (Flanagan et al., 2014; Haapaniemi et al., 2015; Milner et al., 2015; Vogel et al., 2015). Evidences from biochemistry and molecular modeling prove that GOF mutations enhance STAT3 DNA binding affinity, which presumably increases the transcriptional activity of STAT3 (Flanagan et al., 2014).

Upon bacterial or fungal infection, STAT3 is a key innate immune regulator that provides increased numbers of the circulating neutrophils to contain infection. During

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pathogen challenge, endothelial cells secrete increased numbers of G-CSF, the major neutrophil growth factor (Boettcher et al., 2014). Upon the engagement of G-CSF with its receptor, STAT3 is activated in bone marrow granulocytic progenitor cells to stimulate the expression of C/EBPβ and c-Myc. These regulators enhance G_1/S phase progression to drive emergency granulopoiesis (Hirai et al., 2006; Johansen et al., 2001; Zhang et al., 2010). STAT3 can directly enhance the transcriptional activity of C/EBPβ and c-myc (Zhang et al., 2010). Moreover, upregulation of C/EBPβ by STAT3 activation enhance the association of C/EBPβ at the c-myc promoter. The presence of STAT3 and C/EBPβ at the c-myc promoter causes disassociation of the negative regulator C/EBPα from the promoter, hence increasing c-myc transcription (Johansen et al., 2001; Zhang et al., 2010). Mice with STAT3 deletion in hematopoietic cells (e.g. Tek-cre $STAT3^{f/f}$ mice) show defective ability in clearance of *Listeria monocytogenes*. STAT3-deficient neutrophils and macrophages also have impaired ability to mount a bactericidal response, demonstrating the critical roles of STAT3 in pathogen clearance (Matsukawa et al., 2003; Nguyen-Jackson et al., 2010).

STAT3 not only induces neutrophil production to combat pathogen invasion, but also restrains its production and limits inflammation to prevent destructive events (Fielding et al., 2008; Lee et al., 2002). One of the key regulatory mechanisms is through direct induction of the negative regulator SOCS3 (Croker et al., 2004) (more detail later). STAT3 plays an important role in suppressing TLR signal transduction in mature phagocytic cells. It is reported that STAT3-deficient macrophages and neutrophils show enhanced pro-inflammatory cytokine expression upon TLR4 activation (Takeda et al., 1999). STAT3 can inhibit NF-κB function by inducing the expression of

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transcriptional repressors and co-repressors, suggesting an indirect mechanism of STAT3 in restraining inflammation (El Kasmi et al., 2007). In addition, STAT3 was found to directly repress Ube2n gene transcription, which encodes the protein Ubc13, a key E2 ubiquitin-conjugating enzyme. Ubc13 is required for Receptor Activator of NF-κB (RANK) and TLR4 signaling (Zhang et al., 2014b). Therefore, STAT3 exerts a broad anti-inflammatory function either by direct induction of the negative regulator or inhibition of NF-κB or MAPK signaling pathway.

Cellular sources and targets of the IL-10 family cytokines

The expression profiles of the IL-10 family cytokines and their receptors are fundamental for our understanding the biological functions of these cytokines. To this end, Malefyt and colleagues conducted a study to comprehensively analyze the cellular sources and targets of the IL-10 family cytokines by using cDNA libraries obtained from a panel of human hematopoietic cells as well as human tumor cell lines (Nagalakshmi et al., 2004). The expression of the IL-10 family cytokines and receptors was detected by real time quantitative PCR in resting and activated peripheral blood mononuclear cells (PBMCs), T cells, B cell, natural killer cells (NK cells), monocytes, mast cells, and dendritic cells. Their results showed that while monocytes are the major cellular sources for IL-19 and IL-20, a variety of activated immune cells, including monocytes, PBMCs, dendritic cells, and T cells, express IL-10, IL-24, and IL-26; the expression of IL-22 is restricted to activated T cells and mast cells.

For the receptors, IL-10R1 and IL-10R2 were expressed ubiquitously in all the immune cell types examined, indicating hematopoietic cells are the target cells for IL-10. Interestingly, Malefyt group found that while a low expression level of IL-20R2 was

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observed in hematopoietic cells, no expression of IL-20R1 or IL-22R1 was detected in any of the immune cell types, indicating the IL-20 subfamily members, including IL-19, IL-20, IL-22, IL-24, and IL-26, would not be biologically active in hematopoietic cells (Nagalakshmi et al., 2004). However, by examining a panel of stromal and epithelial tumor cells lines, they found that IL-20R1, IL-20R2, and IL-22R1 are highly expressed in epithelial and stromal tissues. Therefore, they concluded the IL-20 subfamily cytokines are produced by immune cells and act on epithelial and stromal cells, bridging the communication between the immune cells and epithelial/stromal cells.

Sabat group further confirms and expands the knowledge of the expression patterns and targets of the IL-20 subfamily cytokines (Kunz et al., 2006). They found that IL-19, IL-20, and IL-24 were not only produced by activated monocytes, but also by keratinocytes to a similar extent. *In vivo* studies demonstrated that these cytokines are preferentially induced in inflammatory tissues. They demonstrated that all the immune cells express IL-20R2, albeit at a lower level compared with IL-10R1 and IL-10R2; the expression of IL-20R1 and IL-22R1 is absent from either resting or LPS activated macrophages, DCs, or other immune cells. In conclusion, IL-10, as a major anti-inflammatory and immunosuppressive cytokine, is exclusively produced by and acts on a variety of immune cells. IL-19, IL-20, and IL-24 can be produced by both immune cells and tissue cells, but only exert their functions on epithelial and tissue cells. IL-22 produced by T cells and IL-26 produced by both monocytes and T cells mediate their effects on epithelial and tissue cells as well (Fig. 14).

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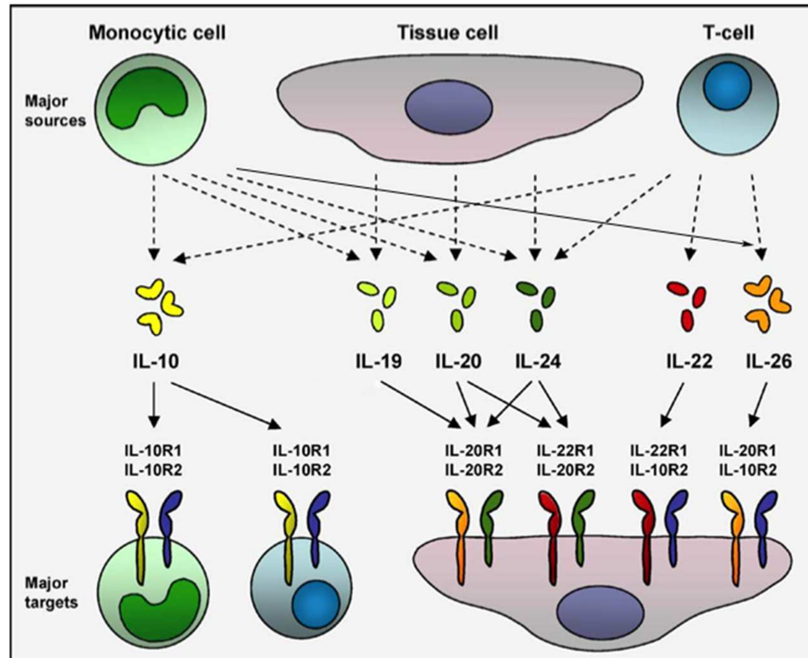

Figure 14 The cellular sources and targets of the IL-10 family cytokines. (Sabat, 2010)

Interleukin-24

In humans, when the MDA-7 gene encodes a protein with an abnormal long signal peptide, it will not be secreted into extracellular space. When the gene encodes a protein with a shorter canonical signal peptide, the protein can be secreted outside the cells and mediates its function through membrane bound receptors. MDA-7 was renamed IL-24 due to its cytokine property (Caudell et al., 2002). The nascent IL-24 protein possesses a long leader sequence to guide the protein into endoplasmic reticulum (ER) for secretion. The leader sequence contains 48 amino acids compared to the conventional 19-22 amino acids in other cytokines. Therefore, IL-24 in extracellular space is actively secreted instead of being a byproduct of cell lysis (Sieger et al., 2004).

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It is reported that the mature IL-24 protein is heavily glycosylated (Caudell et al., 2002; Mhashilkar et al., 2001). In human cell lines, the secreted IL-24 shows a molecular weight of 40 kDa, higher than the intracellular protein with 30/23 kDa. There are three potential N-glycosylation sites in the coding region of IL-24 (Caudell et al., 2002). When MDA-7 protein was treated with glycopeptidase F, an endoglycosidase that cleaves asparagine-linked oligosaccharides at the protein backbone, western blot analysis showed two bands, one at ~23kDa and the other at 18 kDa. When the protein was digested for a longer period time, only one band at 18 kDa was detected, demonstrating that the size of completely deglycosylated mature protein is 18 kDa (Chada et al., 2004b).

Human MDA-7

Since the expression of MDA-7 protein is negatively associated with the progression and metastasis of melanoma cells (Ekmekcioglu et al., 2001; Huang et al., 2001), MDA-7 has been extensively studied in the cancer field. It can selectively induce apoptosis in cancer cells, leaving normal cells unaffected (Ekmekcioglu et al., 2001; Huang et al., 2001; Jiang et al., 1996; Madireddi et al., 2000; Mhashilkar et al., 2001; Saeki et al., 2000; Su et al., 1998). MDA-7 has to be expressed inside cancer cells to induce apoptosis. The induction of apoptosis is related to the upregulation of Bax protein, independent of p53 or retinoblastoma protein (RB) (Jiang et al., 1996; Su et al., 1998). MDA-7 proteins accumulate in the ER and Golgi compartments, cause "ER stress", and subsequently induce apoptosis (Breckenridge et al., 2003; Pahl, 1999). Also, MDA-7 can cause G2/M cell cycle arrest to prevent tumor cells from entering S phase (Ekmekcioglu et al., 2001; Mhashilkar et al., 2001). The induction of reactive

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oxygen species (ROS) in cancer cells has been demonstrated as one of the MDA-7 functions. MDA-7 induces ROS production in mitochondria, thereby causing the release of cytochrome c and loss of mitochondrial transmembrane potential (Breckenridge et al., 2003; Pahl, 1999). In addition, MDA-7 can suppress neovascularization by inhibiting the production of vascular endothelial growth factor (VEGF) in cancer cells (Lebedeva et al., 2007; Ramesh et al., 2003; Sarkar et al., 2007). The mechanisms involved in the anti-cancer effects of MDA-7 are summarized in Fig. 15.

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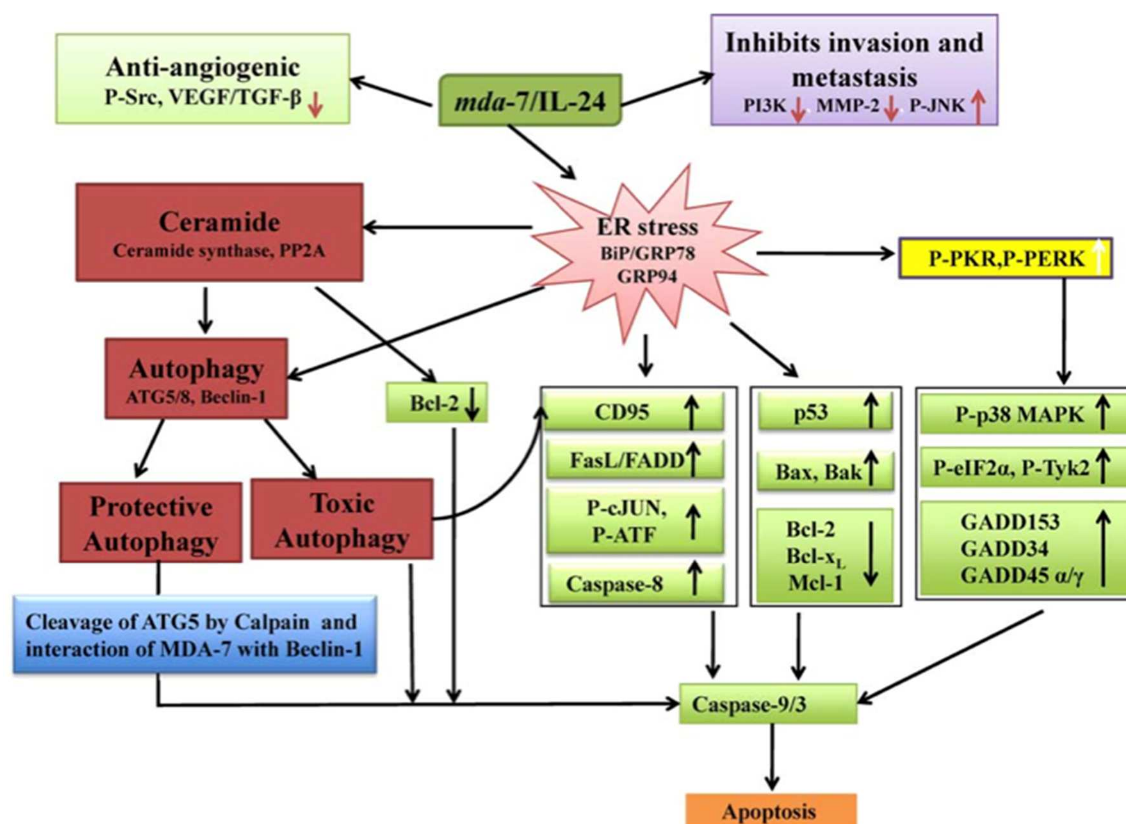

Figure 15 The mechanisms of MDA-7/IL-24 induced apoptosis in cancer cells. (Dash et al., 2010)

Secreted IL-24 is also reported to have anti-cancer effects. It can bind to its receptors to induce cancer-selective cytotoxicity, which is named as an antitumor bystander function of the secreted protein (Chada et al., 2004a). The engagement of secreted IL-24 with its receptors in endothelial cells has been implicated in mediating anti-angiogenesis in cancer tissues (Ramesh et al., 2003). The remarkable effects of IL-24 on cancer cells lead to its evaluation in several clinical trials. In the first clinical trial, IL-24 gene was carried by adenoviruses and directly injected into tumor tissues of patients with advanced carcinoma (Cunningham et al., 2005; Tong et al., 2005). The

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results showed that patients tolerated the injection well. IL-24 was expressed in the injected sites and significantly induced apoptosis in cancer cells. The clinical trials found that the mechanisms of IL-24 function in cancer cells included: induction of apoptosis, antitumor bystander effects, and immune modulation. However, the anti-tumor effect did not last and required repeated IL-24 injections. The clinical trials demonstrated promising results in the application of IL-24 in cancer therapy. However, more research is needed to improve its clinical effects.

Rat c49a/mob-5

In the rat system, two independent groups identified a gene highly homologous to human MDA-7, named as c49a and mob-5 respectively (Soo et al., 1999; Zhang et al., 2000). The gene shares 78% similarity at cDNA level and 68% similarity at amino acid level with human MDA-7 (Soo et al., 1999) (Table 3). The expression of c49a was first reported to be elevated in the leading edge during wound repair. This upregulation was rapid, reaching a peak level within 12-24h, and gradually declining to the baseline level by 14 days (Soo et al., 1999). Spindle-shaped fibroblast-like cells at the wound edge were the major cell types responsible for c49a production. Since elevated c49a protein expression was associated with the proliferation phase of wound repair, c49a was believed to promote cell proliferation instead of inhibiting cell growth. Mob-5 was first cloned as a gene directly induced by oncogenic ras (Zhang et al., 2000). The induction depends on MAP kinase signaling pathway and can be abolished by inhibitors that prevent ras protein membrane targeting. However, mob-5 was only expressed in ras transformed cells, not in non-transformed cell even with the activation of ras signaling. Furthermore, mob-5 receptors can also be directly induced by oncogenic ras in

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transformed cells. This coordinated upregulation of both ligands and receptors by ras oncogene indicates that mob-5 may act on its receptors in an autocrine or paracrine manner to promote cell growth and facilitate cell transformation.

Clearly, rat c49a/mob-5 gene mediates cell proliferation and transformation, whereas human MDA-7 gene has been found to induce apoptosis selectively in cancer cells. The functional discrepancy between these two genes raised a question: how conserved is the gene between species? To answer this question, Liang et al. designed a study to compare the genomic structure and signaling pathway between human MDA-7 and rat c49a/mob-5 (Wang et al., 2004). They found that rodent and human IL-24 genes are truly homologous genes. Both rodent and human IL-24 genes are localized in genomic loci that contain clusters of IL-10, IL-19, IL-20, and IL-24 genes with the same gene arrangement. In addition, both rodent and human IL-24 genes have six exons and five introns, and share conserved exon-intron junctions. The similar coding regions between the two species generate proteins with high sequence homology. Although rodent and human secreted IL-24 are differentially glycosylated, rat mob-5 protein can bind to human IL-24 receptor complex and activate JAK/STAT signaling pathway to promote cell survival and growth (Wang et al., 2004). More importantly, the expression of human IL-24 receptors can be induced by oncogenic ras in human colon cancer cells, demonstrating the consistency between human and rodent IL-24 gene behaviors (Wang et al., 2004). Therefore, human and rodent IL-24 genes are highly homologous. The seemingly functional discrepancy only reflexes the multiple facets of IL-24 functions. Alternatively, the homologs of the gene between species provide foundations for us to evaluate its biological functions in rodents due to the easier genetic manipulation in

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lower class species.

Mouse FISP

The mouse IL-24 ortholog was first identified by representational difference analysis method to isolate genes that are specifically induced by Th2 cells (Schaefer et al., 2001). IL-4 treatment was able to induce the novel gene expression in Th2 cells through STAT6-mediated pathway. Thus, the gene was named IL-4-induced secreted protein (FISP). Subsequent studies revealed that the FISP gene encodes a protein of 220 amino acids with a 65-aa-long potential signal peptide sequence. Blast analysis revealed that FISP shares 89% identity with rat mob-5/c49a and 80% identity with human mad-7 at cDNA level; at protein level, FISP shares 93% similarity with rat mob-5/c49a and 69% similarity with human mad-7 (Table 3). The induction of FISP was only found in Th2 cell, but not Th1 cells, and required both IL-4 receptor and TCR complex signaling. FISP was detected in the conditioned medium of long-term cultured D10.G4 cells, demonstrating it can be actively secreted outside the cells. FISP, as a secreted cytokine in the immune system, indicates it may mediate important roles in host immune response.

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Table 3 Conservation of the primary sequence of IL-24 protein across species. (Wang and Liang, 2005)

			Amino acid sequence homology		
Gene	Species	Alternative name	MDA-7	Mob-5	FISP
hIL-24	Human	MDA-7	100%	68%	69%
rIL-24	Rat	MOB-5, C49A	68%	100%	93%
mIL-24	Mouse	FISP	69%	93%	100%

h, human; m, murine; r, recombinant.

IL-20R cytokines in inflammatory diseases

It is reported that the expression of IL-19, IL-20, and IL-24 (named as "IL-20R cytokines" collectively) is increased in inflamed tissues of various diseases, such as psoriasis, inflammatory bowel disease (IBD), and rheumatoid arthritis (RA) (Andoh et al., 2009; Kragstrup et al., 2008; Kunz et al., 2006). Increased expression of IL-19 and IL-20 mRNA was found in the basal and suprabasal keratinocytes in the epidermis of 80% untreated psoriatic patients, whereas IL-24 mRNA was enriched in mononuclear inflammatory cells in the derma (Romer et al., 2003). In parallel, the expression of IL-20R1, IL-20R2, and IL-22R1 was upregulated in keratinocytes of psoriatic epidermis (Sa et al., 2007). Engagement of IL-20R cytokines with receptors activates STAT3 to induce various downstream gene expression, including proinflammatory cytokines, such as CXCL1, CXCL5, and CXCL7 (Boniface et al., 2005; Sa et al., 2007), genes involved in tissue remodeling, such as MMP1, MMP3, and Kallikreins (Boniface et al., 2005; Li et al., 2009; Liang et al., 2006; Sa et al., 2007; Wolk and Sabat, 2006), and antimicrobial

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peptides, such as S100 family genes and β-defensin (Boniface et al., 2005; Liang et al., 2006; Sa et al., 2007; Wolk et al., 2006). Increased expression of S100A7 (also named as psoriasin) is the characteristic feature of psoriatic skin. Romer et al. reported that the overexpression of IL-19 and IL-20 mRNA in the skin subsided after short-term treatment with cyclosporine A or calcipotriol in psoriatic patients (Romer et al., 2003) Transgenic mice overexpressing IL-20R cytokines further confirm their pathogenic roles in psoriasis (Blumberg et al., 2001) (He and Liang, 2010) (Wolk et al., 2009). IL-20, IL-22 or IL-24 transgenic mice died shortly after birth, with tight, wrinkled, and shiny skin. Histological analysis of the skin revealed epidermal hyperplasia, compact stratum corneum, and abnormal keratinocyte differentiation, hallmarks of psoriasis. Genetic association studies also suggest that IL-20R cytokines are associated with the susceptibility of psoriasis (Kingo et al., 2004; Koks et al., 2004; Koks et al., 2005). Therefore, IL-20R cytokines play an important role in the development of psoriasis, and their up-regulation is a characteristic feature of psoriatic skin.

IL-20R cytokines are upregulated in mononuclear cells in the synovial fluid of patients with rheumatoid arthritis (RA) (Alanara et al., 2010; Kragstrup et al., 2008). IL-24 is also detected in endothelial cells (Kragstrup et al., 2008). The receptors for IL-20R cytokines are found to be expressed in synovial tissues, indicating the involvement of IL-20R cytokines in RA (Kragstrup et al., 2008; Sakurai et al., 2008). Stimulation with IL-19 induced IL-6 production and reduced apoptosis in synovial cells (Sakurai et al., 2008). IL-20 promoted endothelial cell proliferation as well as proinflammatory cytokine expression in synovial cells, such as CCL2, IL-6, and IL-8 (Hsu et al., 2006). In addition, recombinant human IL-20 or IL-24 stimulated the expression of monocyte

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chemoattractant protein 1(CCL2/MCP-1) in cultured synovial fluid mononuclear cells (Kragstrup et al., 2008). Blockage of IL-20R cytokine signaling attenuated disease severity in a rat animal model of collagen-induced arthritis (Hsu et al., 2006). Therefore, IL-20R cytokines induce the expression of proinflammatory cytokines and chemokines in RA, indicating their pathogenic roles.

In contrast to the pathogenic roles of IL-20R cytokines in psoriasis and RA, various studies demonstrated a protective role of these cytokines in IBD. Elevated expression of IL-20R cytokines was found in serum samples of patients with active IBD, but not inactive IBD or healthy controls (Andoh et al., 2009; Dambacher et al., 2009; Fonseca-Camarillo et al., 2014; Fonseca-Camarillo et al., 2013). In a distal colon culture system, when the intestinal epithelial barrier was injured by chemical DSS to induce colitis, colon from IL-19-/- mice elicited more proinflammatory cytokine production than the wild type controls (Azuma et al., 2011). However, the levels of cytokines and chemokines in the steady-state colon organ from IL-19-/- mice were not different from wild type mice, indicating IL-19 modulates the susceptibility and disease severity of colitis. Comparing with the unaffected regions, IL-24 expression was significantly higher in the active regions of ulcerative colitis and Crohn’s disease (Andoh et al., 2009). The inflammatory cytokine IL-1β facilitates the binding of transcriptional factors AP-1 and C/EBP-β to IL-24 promoters to induce IL-24 expression in subepithelial myofibroblasts (SEMFs). IL-24 acts on colonic epithelial cells to induce the expression of SOCS3 and membrane-bound mucins, such as MUC1, MUC3, and MUC4, indicating IL-24 exerts repressive and regulatory effects in mucosal inflammation in IBD (Andoh et al., 2009). The reasons are unknown for the different roles that IL-20R cytokines play in different

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inflammatory diseases. It warrants further research in understanding the physiological and pathophysiological roles of these cytokines in various disease models.

Suppressor of cytokine signaling (SOCS)

As has been mentioned above, excessive inflammation can cause extensive tissue damage or autoimmune diseases. Moreover, any scar formation in the cornea would lead to visual dysfunction. Therefore, cytokine response must be tightly regulated to achieve a balance and restore tissue homeostasis. A negative feedback mechanism has evolved within cells to tune the immune response to prevent destructive events.

Suppressor of cytokine signaling (SOCS) proteins are negative regulators of the JAK-STAT signaling pathway (Dimitriou et al., 2008). The first member of the SOCS family was identified by Yoshimura et al. in 1995 and named as cytokine-induced STAT inhibitor (CIS) (Yoshimura et al., 1995). Subsequent studies identified other proteins that share homologous conformation with CIS. So far, the SOCS family is constituted of eight members: CIS and SOCS1-7 (Minamoto et al., 1997; Naka et al., 1997; Starr et al., 1997). SOCS1 and SOCS3 are the two most studied members in the SOCS family. All SOCS proteins contain a central SH2 domain and a SOCS box (Babon et al., 2009; Zhang et al., 1999) (Fig. 16). Because of the SH2 domain, any tyrosine phosphorylated signaling protein (p-receptor, p-JAK, or p-STAT) is a conceivable substrate. The SH2 domain acts as an adaptor to recruit ubiquitin ligases to the phosphorylated signaling proteins, facilitating their degradation. SOCSs bind to the E3 complex through the conserved SOCS box. SOCSs are also pseudo-substrate inhibitors for JAKs and their STAT proteins, thereby terminating signal transduction (Dimitriou et al., 2008). Additionally, SOCS1 and SOCS3 have a kinase inhibitory region that can inhibit JAKs

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directly (Sasaki et al., 1999; Yasukawa et al., 1999). While SOCS1 negatively regulates STAT1 activation in the IFN-γ signaling pathway, SOCS3 is a major inhibitor in the STAT3 signaling cascade (Kubo et al., 2003; Yoshimura et al., 2007).

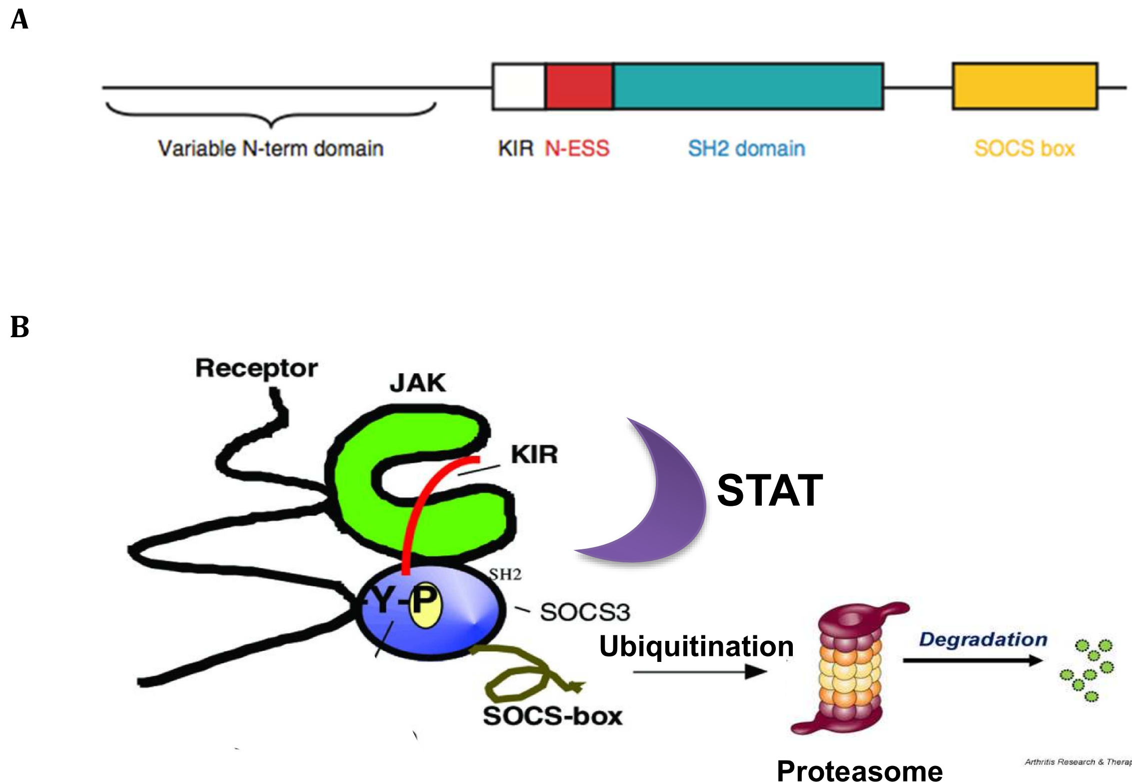

Figure 16 The structure and functions of the SOCS family. (A) The proteins of the SOCS family all contain a SH2 domain and a SOCS box. Additionally, SOCS1 and SOCS3 have a kinase inhibitory region (KIR). (B) The SH2 domain can bind to any tyrosine phosphorylated signaling protein (p-receptor, p-JAK, or p-STAT) and target the binding partners to ubiquitination and proteasome degradation. SOCS proteins bind to the E3 complex through the conserved SOCS box. KIR can directly inhibit JAK activity. (Dimitriou et al., 2008)

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The SOCS3-mediated negative feedback loop plays an important role in maintaining tissue homeostasis, disruption of which would cause unrestrained inflammation or increased infection susceptibility. It is reported that SOCS3 expression is increased in inflamed human tissues, particularly in recruited leukocytes and also in epithelia (White et al., 2011). SOCS3 expression is enhanced in both acute and chronic inflammation, with a higher level in acute inflammation (White et al., 2011). Uto-Konomi et al. reported that keratin 5-specific SOCS3 conditional knockout (cKO) mice displayed epidermal hyperplasia and massive leukocyte infiltration. Further mRNA detection revealed that the expression of IL-1β, IL-4, IL-6, IL-19, IL-20, and IL-24 was increased in SOCS3 cKO skin tissue. The authors concluded that SOCS3 negatively regulates STAT3 hyper-activation to prevent skin inflammation (Uto-Konomi et al., 2012).

SOCS3 expression is not only stimulated by cytokines, but also by pathogen components that activate the innate immune response (Carow and Rottenberg, 2014). Some pathogens have evolved to induce the expression of SOCS3 to suppress host inflammatory response, facilitating their invasion in the host. It is known that several viruses can stimulate SOCS3 expression, such as human *respiratory syncytial virus* (RSV) (Hashimoto et al., 2009), *hepatitis C virus* (Collins et al., 2014), *herpes simplex virus* (Yokota et al., 2005), and *Epstein-Barr virus* (Michaud et al., 2010). It is believed that increased SOCS3 expression suppresses type I IFN production, and hence attenuates the anti-viral response (Carow and Rottenberg, 2014). Silencing of SOCS3 by siRNA inhibits viral replication (Hashimoto et al., 2009; Yokota et al., 2005). The roles of SOCS3 in bacterial and parasitic infections are less clear and warrant more research. Enhanced SOCS3 expression in myeloid cells conveyed resistance to *M.*

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tuberculosis through the regulation of the IL-6/STAT3 signaling pathway (Carow et al., 2013). SOCS3$^{fl/fl}$ lck cre mice showed increased susceptibility to *Leishmania major* due to over-production of the anti-inflammatory cytokines IL-10 and TGF-β. On the other hand, SOCS3 transgenic mice (Lck-SOCS3 Tg mice) that over express SOCS3 in T cells also succumbed to *L. major* infection because of the increased IL-4 secretion (Kinjyo et al., 2006; Nakaya et al., 2011). Therefore, SOCS3 expression must be tightly regulated in infection scenarios to ensure a favorable outcome.

Central hypothesis

Our central hypothesis is that IL-24 is an early response cytokine that modulates corneal innate immune response and involved in the pathogenesis of microbial keratitis in a SOCS3 related manner. This hypothesis will be tested with three specific aims:

Aim 1: To characterize the expression of IL-24 and SOCS3 in the cornea in response to *PA* infection. Experiments in this aim will identify the expression pattern of IL-24 and SOCS3 in mouse corneal epithelial cells as well as whole cornea after *PA* infection *in vivo*. We will also determine the expression of IL-24 receptors in the cornea to identify IL-24 targeted cells. By using MyD88-/- mice, we will explore whether the expression of IL-24 and SOCS3 is MyD88 dependent.

Aim 2: To evaluate the functions of IL-24 and SOCS3 in *PA* keratitis. The experiments in this aim will explore the roles that IL-24 and SOCS3 play in corneal innate immunity in response to *PA* infection by using siRNA, recombinant protein, and receptor blockage approaches. Endpoints include the clinical outcome, bacterial clearance, and cellular infiltration. Moreover, we will determine how loss and gain of

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function of IL-24 modulate the expression of pro- and anti-inflammatory cytokines in the cornea.

Aim 3: To explore the regulatory relationship of IL-24 and SOCS3 *in vitro* with primary human corneal epithelial cells (HCECs). Experiments in this aim will use heat-killed *PA* to challenge primary HCECs to determine whether IL-24 is also induced in HCECs upon *PA* challenge. In addition, we will use recombinant proteins to treat cells to explore the cytokine regulation network.

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CHAPTER 1: CHARACTERIZE THE EXPRESSION OF IL-24, ITS RECEPTORS, AND SOCS3 IN THE CORNEA IN RESPONSE TO *P. AERUGINOSA* INFECTION.

Summary

We propose that *PA* induces early expression of IL-24 and SOCS3 in the cornea through MyD88 signaling pathway, and IL-24 targets corneal epithelial cells. Our cDNA array results demonstrate that IL-24 and SOCS3 are induced at the early stage of *PA* infection (6hpi) in corneal epithelial cells. *PA* activates TLRs in corneal epithelial cells. The signal may be transduced through MyD88 to directly induce early IL-24 expression. As SOCS3 is a downstream negative regulator of IL-24, SOCS3 would share a similar expression pattern with IL-24. Since both Malefyt's and Sabot's groups reported that IL-24 receptor chains are absent in immune cells (Kunz et al., 2006; Nagalakshmi et al., 2004), IL-24 may exert its function primarily in corneal epithelial cells.

In this study, we used the needle scratch model to induce *PA* infection in B6 mouse cornea. Our cDNA microarray results show that IL-24 and its downstream negative regulator SOCS3 are differentially regulated by flagellin pretreatment. Since flagellin pretreatment is known to induce profound innate immune protection, down-regulation of IL-24 and SOCS3 implies negative effects of IL-24 and SOCS3 on the outcome of microbial keratitis. To define the role of the IL-24-STAT3-SOCS3 signaling pathway in *PA* keratitis, it is of importance to first verify the expression patterns of IL-24 and SOCS3, as well as the IL-24 receptor subunits at both mRNA and protein levels. By using quantitative PCR analysis, we confirmed that elevated expression of IL-24 and SOCS3 in response to *PA* infection was dampened by flagellin pretreatment. Furthermore, time course study in whole corneal extracts demonstrated that the expression pattern of SOCS3 follows the same kinetics as that of IL-24, indicating the

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correlation of IL-24 and SOCS3. The expression of both IL-22R1 and IL-20R2 was detected in corneal epithelial cells, demonstrating IL-20R cytokines target corneal epithelial cells. The absence of IL-20R2, the common chain of the receptor complex, in bone marrow derived macrophages indicates IL-20R cytokines may not act on macrophages. By recruiting the adapter protein MyD88, TLR signals are transduced into nucleus to activate inflammatory response to combat pathogen invasion (Kawai and Akira, 2007). Our results demonstrate that MyD88 plays an important role in generating inflammatory response in the cornea in response to *PA* infection. MyD88 signaling pathway accounts for the majority of the induction of IL-24 and SOCS3 at the early stage of infection.

Introduction

The corneal epithelial layer forms the first line of defense against pathogens. TLRs recognize bacterial components to induce pro-inflammatory and anti-inflammatory cytokine expression in an attempt to clear pathogens (Pearlman et al., 2008). MyD88 is a convergent adaptor for many TLRs, including TLR4 and TLR5. Activation of MyD88 eventually results in phosphorylation of NF-κB, which would be translocated into the nucleus to promote the expression of cytokines and chemokines (Kawai and Akira, 2007). A certain amount of inflammatory response is beneficial to pathogen clearance; however, prolonged unrestrained inflammation is devastating and contributes to corneal destruction (Thakur et al., 2002). Therefore, the TLR-mediated inflammatory response must be precisely regulated.

IL-1β is the major inflammatory cytokine in response to infection. Signaling via TLRs can directly induce IL-1β expression, which can act as a chemokine to recruit

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immune cell infiltration (Karmakar et al., 2012; Rudner et al., 2000). Neutrophils can further produce a large amount of IL-1β, resulting in a cascade of inflammation (Karmakar et al., 2012). In response to infection, γδ T cells can produce IL-17A, another important chemokine to recruit neutrophils (Cho et al., 2010). Matrix metalloproteinase 13 (MMP13) is an enzyme that participates in collagen degradation. MMP13 is considered to be an important inflammatory cytokine since it can facilitate pathogen dissemination (Gao et al., 2015). Besides the inflammatory cytokines, TLR signaling also induces the expression of anti-inflammatory cytokines. IL-1 receptor antagonist (the gene is referred as IL-1RN; the encoded protein is referred as IL-1RA) can be secreted by epithelial cells, as well as a variety of immune cells in response to infection. It can bind to cell surface IL-1 receptor to terminate signal transduction, functioning as a natural inhibitor of IL-1β (Weber et al., 2010). S100A8 and A9 belong to the S100 family of calcium-binding proteins. The heterodimer of S100A8 and A9 (also referred to as calprotectin) plays critical roles in innate immunity since it can prevent the growth of bacteria, fungi, and protozoa by chelating Mn^{+2} and Zn^{+2} ions (Clark et al., 2016; Clohessy and Golden, 1995; Damo et al., 2013; Sohnle et al., 2000). Besides their antimicrobial properties, S100A8/A9 are also associated with inflammation, as evidenced by their high expression levels in psoriasis (Sa et al., 2007).

Our previous studies showed that pretreatment of mouse corneas with flagellin, which signals through TLR5, can protect mouse corneas from *PA* infection (Kumar et al., 2010; Kumar et al., 2008; Yoon et al., 2013). We found that this flagellin-induced protection is due to the reprograming of gene expression in epithelial cells to enhance the innate defense against invasive pathogens (Gao et al., 2013). Our genome-wide

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cDNA microarray showed that the expression of IL-24 and its downstream negative regulator, SOCS3, was notably upregulated in response to infection and significantly suppressed by flagellin pretreatment (Gao et al., 2013).

Increased expression of IL-19, IL-20, and IL-24 (IL-20R cytokines) was found in various inflammatory tissues, such as the skin of psoriasis, and the mucosa of IBD patients, indicating these cytokines may play an important role in inflammation (Andoh et al., 2009; Kragstrup et al., 2008; Kunz et al., 2006). The expression of IL-19 and IL-24 was increased in mouse skin in response to Methicillin-resistant *Staphylococcus aureus* (MRSA), while the expression of IL-20 remained unchanged during the course of infection (Myles et al., 2013). It is reported that a high level of SOCS3 expression was found in the epithelia of human tissue with acute or chronic inflammation, especially with acute inflammation (White et al., 2011). Besides, some pathogens can induce SOCS3 expression to evade the immune response (Carow and Rottenberg, 2014). However, the expression patterns of IL-20R cytokines and SOCS3 in the cornea in response to *PA* infection remain unknown.

Results

***PA* infection induces IL-24 and SOCS3 expression in mouse corneal epithelial cells (CECs)**

To verify the cDNA microarray results, we infected mouse corneas with 1.0×10^{4} colony forming units (CFUs) of *PA* stain *ATCC 19660*. Corneal epithelial cells were collected at 6hpi and subjected to PCR analysis. As shown in Figure 17A, flagellin challenge alone slightly induced IL-24 expression relative to the naïve cornea. *PA*-infection increased the expression of IL-24, and this elevation of IL-24 expression was dampened by flagellin pretreatment in B6 mouse CECs. As IL-24 shares receptors with

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IL-19 and IL-20, we explored the induction of these cytokines in response to infection. While low levels of IL-19 were detected in all samples, no IL-20 mRNA was detected. The infection-induced expression of SOCS3 was also dampened by the flagellin pretreatment (Fig. 17B). The expression of IL-1β, an innate immune and major inflammatory mediator, significantly increased after *PA* infection. IL-1β expression was attenuated to an extent equivalent to the normal control, consistent with flagellin-induced protective and anti-inflammatory response (Fig. 17B). There is no significant change in IL-6 level at 6 hpi in mouse CECs, with or without flagellin pretreatment (Fig. 17B).

The induction of IL-24 and SOCS3 in mouse CECs after *PA* infection was further confirmed at the protein level. Mouse CECs were collected at both 6hpi and 18hpi for immunoblot analysis. As shown in Fig.17C and E, *PA*-infection induced IL-24 protein expression as early as 6hpi. The abundance of IL-24 protein further increased at 18hpi. The activation of STAT3 followed a similar expression pattern as that of IL-24. SOCS3 protein was detected at a later time point, 18hpi (Fig. 17D and E).

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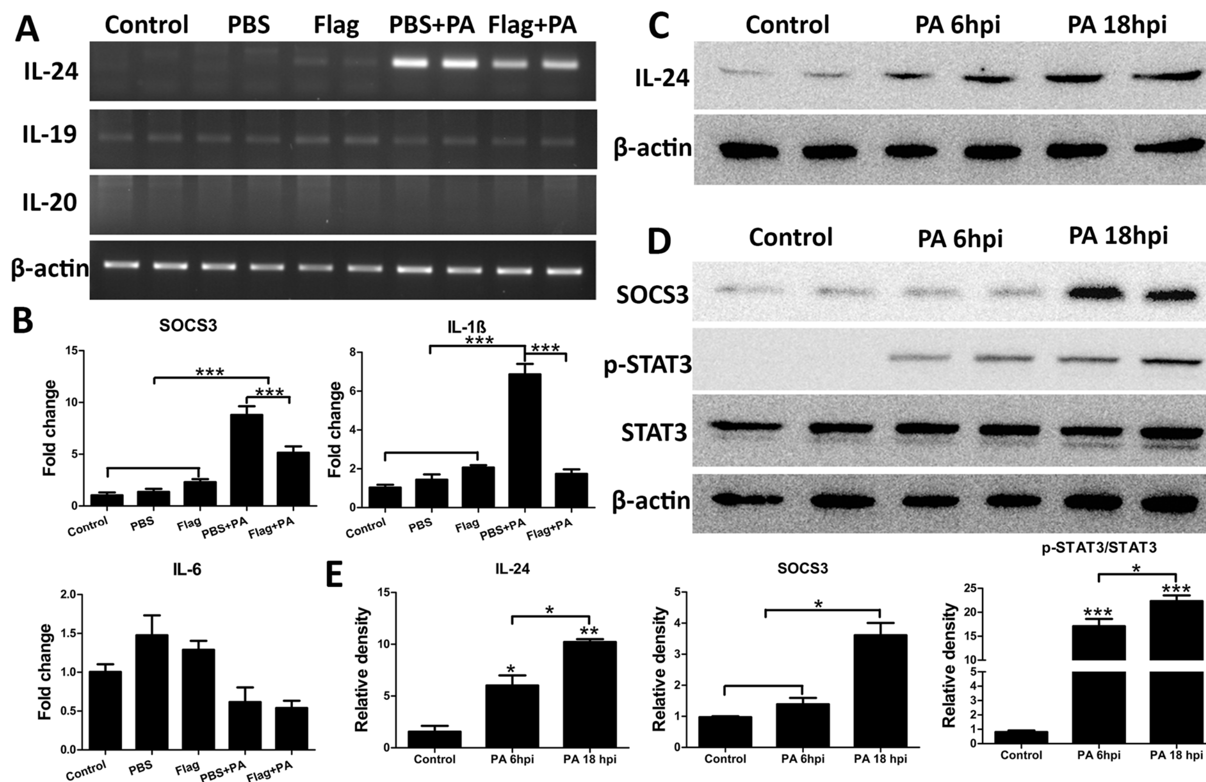

Figure 17 *P. aeruginosa* (*PA*) infection induces IL-24 and SOCS3 expression in B6 mouse corneal epithelial cells; Flagellin pretreatment attenuates their expression. Mouse corneas were scratched with a needle and topically pretreated with 500ng flagellin for 24 hours. PBS was used as control. Then the corneas were scratched again and inoculated with *PA* (*ATCC 19660*, CFUs: $1.0X10^4$). Cornea epithelia were collected at 6 hpi for semi-quantitative RT-PCR (A) or quantitative real-time PCR (B) analysis of IL-24, IL-19, and IL-20 (A), SOCS3, IL-1β, and IL-6 (B). Results are presented relative to those of naïve corneas, set as 1. Mouse corneas were challenged with *PA*, then corneal epithelia were collected at 6 hpi and 18 hpi for immunoblot analysis of IL-24 (C), SOCS3, p-STAT3, and STAT3 (D). β-actin serves as the loading control. (E) Quantification of protein levels based on the densitometry of the Western blots in C and D by using the software Carestream MI SE (Informer Technologies, Inc.). P values were generated by one-way ANOVA. Stars on top of columns are P value results comparing with control. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Data are representative of three independent experiments (B: mean + s.e.m.). Flag: flagellin. The time point in (A) and (B) was used as the same time point with the cDNA array to confirm the cDNA array results. One more time point in (C) (18 hpi) was used to explore the trend of protein expression level as the infection continued.

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IL-24, SOCS3, and IL-1β are early responsive genes upon *PA* infection

Having shown the effects of infection and flagellin pretreatment on cytokine expression, we then performed a time course study to compare the expression of IL-24 with IL-1β, SOCS3 and IL-6 in response to *PA* infection. The expression of IL-1β was induced and detectable at 3hpi, and continued to increase gradually as the infection progressed. Two peaks of IL-24 up-regulation were observed: a rapid and marked increase at 3hpi, followed by a lower but significantly elevated level at 6hpi, and then a gradual increase to a higher level at 18hpi (Fig. 18A). The expression of SOCS3 followed the observed early and late kinetics of IL-24. The expression of IL-6 started at a later time, 9 hpi, and reached a significantly higher level at 18hpi, compared to the naïve cornea (Fig. 18A). Hence, the infection-induced expression of SOCS3 is correlated to that of IL-24, but not IL-6, which is also known to activate the JAK/STAT3 pathway to induce SOCS3 expression. Up-regulation of IL-19 was only detected at a later stage of infection, 18 hpi. No elevated expression of IL-20, IL-22 or SOCS1 was detected at all the time course studied. Significant elevation of IL-17A was detected at 18 hpi (Fig. 18A).

The expression of some of these cytokines and the activation of STAT3 were also assessed by using Western blotting or ELISA in whole corneal extracts. Consistent with the mRNA expression pattern, IL-24 protein expression was markedly increased at the early stage of infection, 3 hpi, followed by a significant subsidence at 6 hpi and a gradual increase at 9 hpi and 18 hpi. The activation of STAT3 detected by the phospho-STAT3 level followed a similar expression pattern with that of IL-24. SOCS3 protein expression was increased at 3 hpi and 6 hpi. The subsidence of SOCS3 protein lagged

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behind that of IL-24 and occurred at 9 hpi, followed by an increase to a higher level at 18 hpi (Fig. 18B and C). The protein levels of the inflammatory cytokines IL-1β and IL-6 increased, with higher levels observed at 18 hpi than at 9 hpi (Fig. 18D).

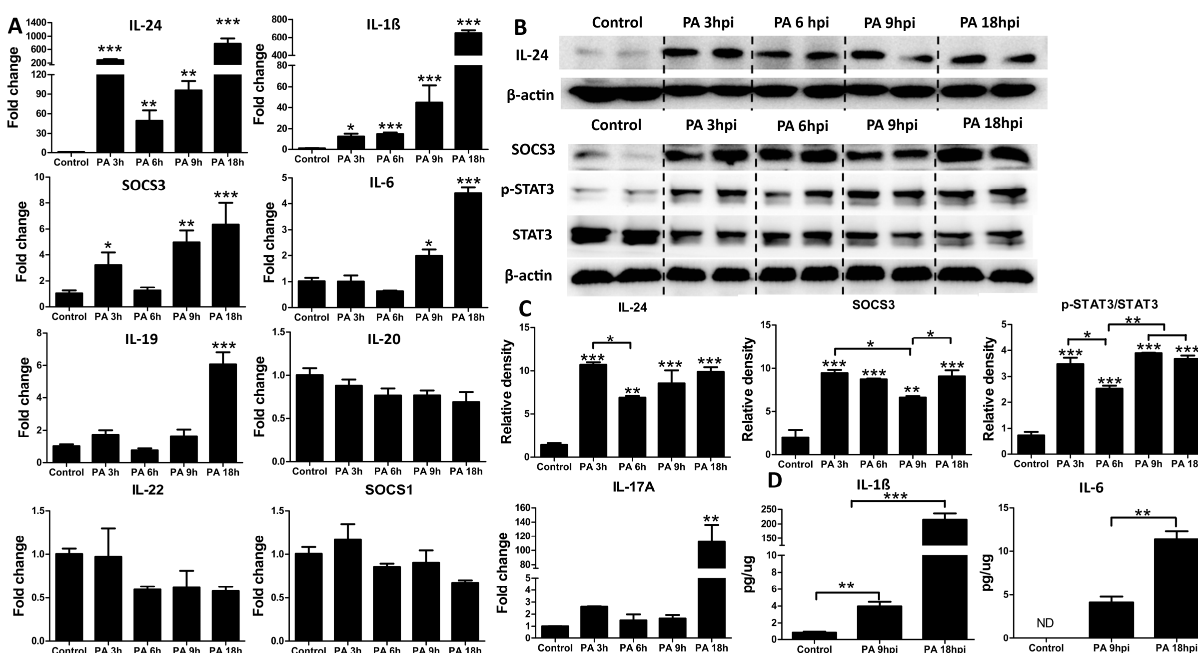

Figure 18 IL-24, SOCS3, and IL-1β are early responsive genes after *PA* infection in B6 mouse cornea. Mouse corneas were infected with *PA* as in Fig. 1. Whole corneas were collected at 3 hpi, 6 hpi, 9 hpi, and 18 hpi for qPCR analysis of IL-24, IL-1β, SOCS3, IL-6, IL-19, IL-20, IL-22, SOCS1, and IL-17A (A). Results are presented relative to those of naïve corneas, set as 1. (B) Immunoblot analysis of IL-24, SOCS3, p-STAT3, and STAT3 in cell lysates of the whole corneas infected with *PA* for 3h, 6h, 9 h and 18 h. β-actin serves as the loading control. (C) Quantification of protein levels based on the densitometry of the Western Blots in B. (D) ELISA assays of IL-1β and IL-6 in cell lysates of the whole corneas infected with *PA* for 9 h and 18 h. Stars on top of columns are P value results comparing with control. *P < 0.05, **P < 0.01, and ***P < 0.001 (ANOVA). Data are representative of three independent experiments (A and C: mean + s.e.m.). ND: not detected.

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The receptor expression of IL-20R cytokines in mouse cornea

The expression patterns of the receptors for IL-20R cytokines remain unknown in the cornea in response to *PA* infection. PCR analysis in whole corneal extracts revealed significant decrease in the expression of the common chain IL-20R2 at 3 hpi, followed by further decrease as the infection progressed. Significant increase of IL-22R1 was detected at 18 hpi. There is no significant change in the expression of IL-20R1 in the course of *PA* infection (Fig. 19A). Previous reports based on PCR studies demonstrated that the receptors for IL-20R cytokines are mainly expressed in epithelial cells, but not in immune cells (Kunz et al., 2006; Nagalakshmi et al., 2004). We further assessed the receptor expression in protein level in membrane protein enriched fraction. Mouse corneas were infected with *PA*; corneal epithelial cells were collected at 18 hpi for immunoblot analysis of the receptors (Fig. 19C and D). While equal levels of IL-22R1 were detected in both the naïve and infected CECs at around 95 kDa, the abundance of IL-20R2 proteins were decreased in the infected CECs, comparing with the naïve control. The expression patterns in protein levels in CECs are consistent with the mRNA levels in whole corneal extracts, indicating CECs may be the predominant sources of the receptors. As in immune cells, SOCS3 is mainly produced by macrophages and is the downstream mediator of IL-20R cytokines (Qin et al., 2012; Uto-Konomi et al., 2012), mouse bone marrow cells were isolated and differentiated into macrophages to evaluate the receptor expression patterns in immune cells. Flow cytometry in Fig. 19B demonstrates the bone marrow derived cells were successfully differentiated into macrophages, expressing both CD11b and F4/80 antigens. Immunoblot analysis shows no bands were detected when the membrane was probed with IL-20R2 antibody,

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whereas IL-22R1 was detected in ~60 kDa area, with a decreased expression level in heat-killed *PA* challenged macrophages, comparing with the unchallenged control (Fig. 19C and D). In sum, our results confirm that IL-20R cytokines mainly act on epithelial cells; the expression of the common IL-20R2 chain decreases in response to *PA* infection in mouse CECs.

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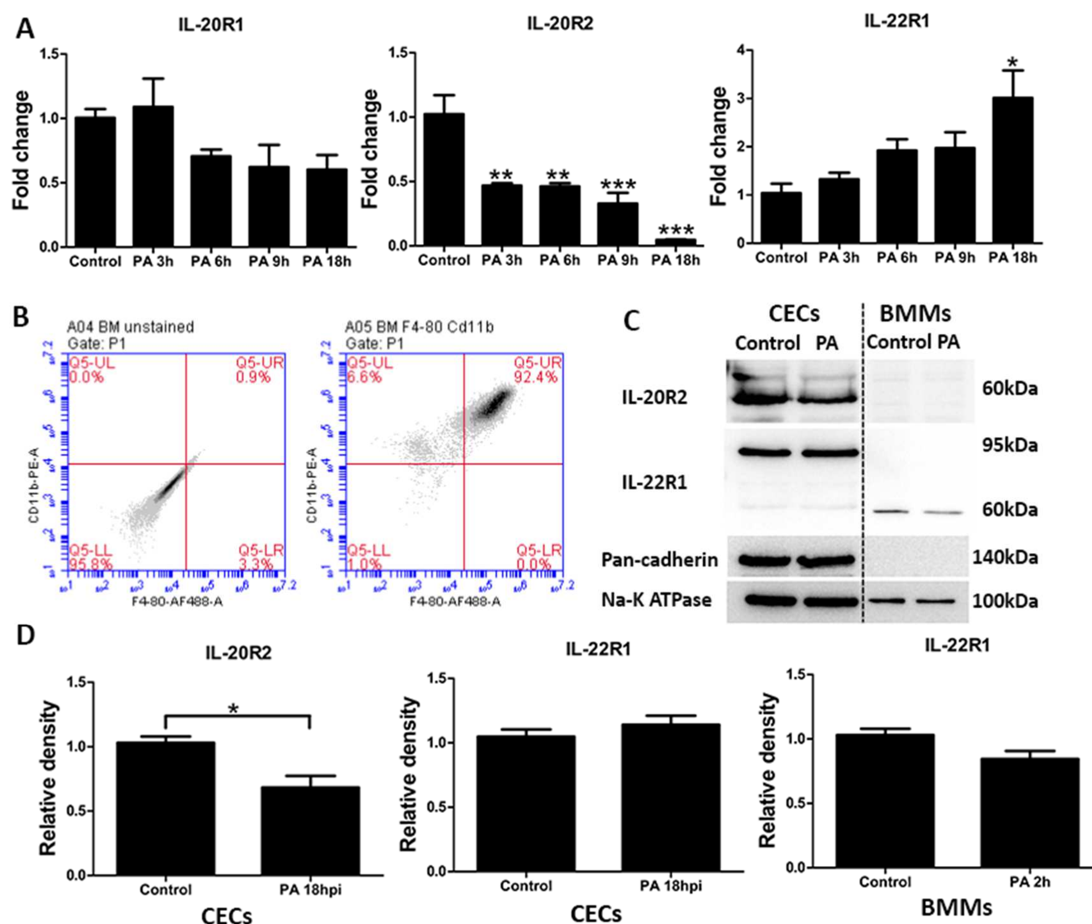

Figure 19 The receptor expression of IL-20R cytokines in mouse cornea. Mouse corneas were infected with *PA* as in Fig. 1. Whole corneas were collected at 3 hpi, 6 hpi, 9 hpi, and 18 hpi for qPCR analysis of IL-20R1, IL-20R2, and IL-22R1 (A). Results are presented relative to those of naïve corneas, set as 1. (B) Mouse bone marrow cells were isolated and differentiated into macrophages in the presence of mouse recombinant macrophage colony stimulating factors (M-CSF, 20 ng/ml) for 7 days. Flow Cytometry was used to analyze the expression of Mac-1 and F4/80 surface antigens to confirm the cell identity. (C) Immunoblot analysis of IL-20R2, IL-22R1, Pan-cadherin, and Na-K ATPase in cell lysates of the corneal epithelial cells (infected with *PA* for 18 h) and bone marrow derived macrophages (challenged with heat-killed *PA* for 2h). Pan-cadherin and Na-K ATPase serve as the loading control. (D) Quantification of protein levels based on the densitometry of the Western blots in C. Relative density was based on the densitometry of Na-K ATPase. Stars on top of columns are P value results comparing with control. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (ANOVA). Data are representative of three independent experiments (A: mean + s.e.m.). CECs: Corneal epithelial cells; BMM: Bone marrow derived macrophages.

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The expression of IL-24 and SOCS3 is MyD88 dependent

Since MyD88 is a common adaptor for the membrane-bound TLRs, we used MyD88-/- mice to evaluate whether the induction of IL-24 and SOCS3 in response to *PA* infection depends on MyD88 signaling pathway. Age-match wild type and MyD88-/- mice in B6 background were infected with *PA* in the corneas. The corneas were examined and photographed at 18 hpi to evaluate the severity of keratitis. As shown in Fig. 20A, around 25% of the cornea in wild type mice was inflamed and opaque. However, the corneas in MyD88-/- mice displayed only slight cloudiness and appeared to be comparatively less inflamed.

Whole corneal samples were collected at both 6 hpi and 18 hpi to quantify the expression of IL-24, SOCS3, pro- and anti-inflammatory cytokines in wild type and MyD88-/- mice (Fig. 20B). At 6 and 18 hpi, there was significantly less IL-24 and SOCS3 in infected MyD88-/- corneas than wild type corneas. In MyD88-/- corneas, the level of IL-24 appeared the same at 6 hpi and 18 hpi, whereas SOCS3 expression was significantly higher at 18 hpi than 6 hpi. The inflammatory cytokines IL-1β and MMP13 were also significantly reduced in MyD88-/- corneas comparing to wild type corneas at both time points examined. Significantly more IL-1β was observed at 18 hpi comparing to 6 hpi in MyD88-/- corneas, whereas MMP13 expression was higher at 6 hpi than 18 hpi. As demonstrated earlier, no significant elevation of IL-6 or IL-17 was detected at 6 hpi in wild type corneas. At 18 hpi, their expression was significantly lower in MyD88-/- corneas compared to wild type corneas. Elevated expression of IL-1RN and S100A8/A9 was observed in infected wild type corneas at 6 hpi, with significantly higher level at 18 hpi. Although there was no significant decrease of IL-1RN at 6 hpi in MyD88-/- corneas,

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less IL1-RN was detected at 18 hpi in MyD88-/- corneas than wild type mice. The expression of S100A8 and A9 was significantly reduced in MyD88-/- corneas compared to wild type corneas at both time points. Overall, these results demonstrate decreased levels of IL-24, SOCS3, pro- and anti-inflammatory cytokines in MyD88-/- corneas in response to *PA* infection, which correlates with less inflammation seen in MyD88-/- corneas under the microscope. The expression of IL-24 and SOCS3 partially depends upon MyD88 signaling pathway.

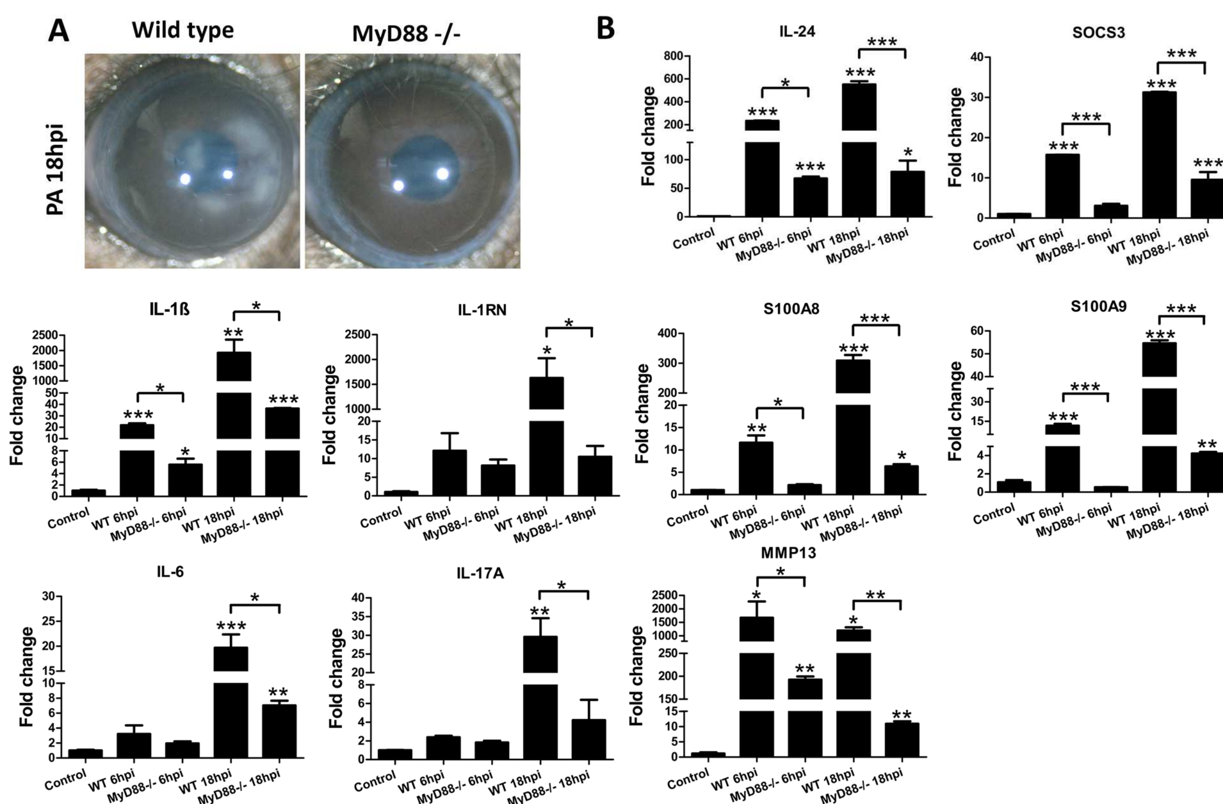

Figure 20 The expression of IL-24 and SOCS3 is MyD88 dependent. (A) MyD88-/- mice in B6 background were infected with *PA* in the corneas. Age-match wild type B6 mice were used as control. The corneas were photographed at 18 hpi. (B) Whole corneal samples were collected at 6 hpi and 18 hpi for PCR analysis of IL-24, SOCS3, IL-1β, IL-1RN, S100A8, S100A9, IL-6, IL-17A, and MMP13. Results are presented relative to those of wild type naïve corneas, set as 1. Stars on top of columns are P value results comparing with control. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (ANOVA). Data are representative of three independent experiments (B: mean + s.e.m.).

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Discussion

These studies verify our cDNA results, demonstrating IL-24 and SOCS3 are induced by *PA* infection and the elevated expression is dampened by flagellin pretreatment in mouse CECs. Among the IL-20 subfamily cytokines, IL-24 is the early responsive gene in mouse cornea in response to *PA* infection; IL-19 appears late in the course of infection; no elevated IL-20 or IL-22 was detected in the cornea post *PA* infection. The expression of both R1 and R2 receptor chains in CECs suggests that IL-24 mainly acts on these residential cells.

Our previous studies demonstrate that flagellin pretreatment is able to protect mouse corneas from bacterial or fungal infection (Gao et al., 2011; Kumar et al., 2010; Kumar et al., 2008; Liu et al., 2014; Yoon et al., 2013). Theoretically speaking, flagellin can be used as a prophylactic agent to prevent potential microbial infection in high-risk individuals, such as patients who have scratched corneal epithelia after contact lens wear. However, flagellin, as a protein, would promote antibody production to neutralize its effects. In addition, the effects and side effects of repeated application on the ocular surface have not yet been tested (Campodonico et al., 2010; Prince, 2006). In order to take advantage of flagellin-induced protection for therapeutic purposes, we investigate the genes that are differentially regulated following flagellin pre-treatment, harboring the thoughts to find potential therapeutic reagents to control infection and inflammation.

Innate immunity forms the first line of defense upon pathogen invasion. TLRs detect pathogen components and recruit MyD88 adaptor to mount immune response to combat pathogens (Kawai and Akira, 2007). The corneas in MyD88-/- mice developed less inflammation during the early stage of infection, demonstrating the critical role of

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MyD88 in response to corneal infection. Our study is consistent with the previous report stating that reduced intraocular inflammation was observed in MyD88-/- mice with *B. cereus* endophthalmitis, comparing to wild type B6 mice (Parkunan et al., 2015). Infected MyD88-/- corneas produced significantly less pro-inflammatory cytokines, such as IL-1β, MMP13, IL-6, IL-17A, and less anti-microbial molecules, such as IL-1RN, S100A8 and A9, than infected wild type corneas in an early stage of infection. The expression of IL-24 and SOCS3 also depends on MyD88 signaling pathway, as less IL-24 and SOCS3 were detected in MyD88-/- corneas. Significantly elevated expression of IL-24 and SOCS3 in infected MyD88-/- corneas compared to naïve corneas demonstrates that other pathways may be involved in the induction of IL-24 and SOCS3.

While the expression of IL-1β in response to infection increased with time, at the mRNA level, IL-24 had two peaks: one at the early stage of infection, 3 hpi, followed by a subsidence at 6h, and then a gradual increase to another peak at 18hpi. Gradual increase in IL-1β expression may be related to the increased inflammatory response and bacterial burden in the cornea. IL-1β, as the master regulator of the innate immune response, may contribute to innate defense and inflammation. On the other hand, IL-24 may be regulated first as a downstream mediator of TLR signaling, similar to IL-1β. Unlike IL-1β, which is capable of regulating its self-expression in a positive amplification loop, the TLR-mediated expression of IL-24 declined within the first few hours. The rise of IL-1β or other cytokines may stimulate the expression of IL-24, resulting in a gradual increase following the initial elevation. Elevated levels of IL-24 may balance the strong inflammatory response of the corneas as infection progresses.

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IL-20 subfamily members share the JAK/STAT3 pathway with IL-6. In our study, *PA* infection induced early expression of IL-24 in mouse cornea. The mRNA expression of IL-6 was not detected until a later time (9 hpi) in the course of *PA* infection. Activation of STAT3 was detected at an early time point, corresponding to the expression of IL-24. We suggest that the early activation of STAT3 is due to the expression of IL-24. Lack of elevated SOCS1 expression in infected corneas demonstrates that SOCS3 is the major downstream mediator of IL-20R cytokine signaling pathway in mouse cornea.

Expression of both IL-22R1 and IL-20R2 was observed in mouse corneal epithelial cells, indicating IL-20R cytokines target corneal epithelial cells. Andoh et al. also reported that IL-20R1, IL-22R1, and IL-20R2 are expressed in epithelial cells from both normal colonic mucosa and colonic mucosa with IBD (Andoh et al., 2009). Decreased expression of IL-20R2 in infected corneas may reflect feedback inhibition of receptor expression due to the massive production of ligands, which would be IL-24 and/or IL-19. Lower molecular size of IL-22R1 in macrophage extracts (60 kDa comparing to 95 kDa in corneal epithelial extracts) indicates that the receptor may be chemically modified differently between the two cell types. No IL-20R2 was detected in macrophage extracts with or without heat-killed *PA* challenge, indicating that IL-20R cytokines cannot target macrophages. Our study is consistent with Carollo and colleagues' report showing that R1 receptors for IL-20R cytokines have a wide expression pattern, whereas IL-20R2 expression is more restricted to certain cell types (Blumberg et al., 2001). However, by using PCR analysis of a wide range of immune cells, both Malefyt and Sabat group demonstrated that no expression of IL-20R1 or IL-22R1 in immune cells; IL-20R2 was expressed at a low level in immune cells (Kunz et

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al., 2006; Nagalakshmi et al., 2004). The reason for the disparity of these studies is unknown and warrens more research. Nevertheless, a common conclusion can be reached that IL-20R cytokines do not act on immune cells.

In summary, we demonstrate that IL-24 is an early responsive gene in mouse cornea post *PA* infection. The expression pattern of SOCS3 correlates with that of IL-24, indicating it may mediate the downstream effect of IL-24. The induction of both IL-24 and SOCS3 partially depends on MyD88 signaling pathway. The restricted expression of receptors on corneal epithelial cells indicates IL-24 acts on corneal epithelial cells, but not immune cells.

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CHAPTER 2: EVALUATE THE FUNCTIONS OF IL-24 AND SOCS3 IN *PA* KERATITIS.

Summary

We have shown that IL-24 and SOCS3 are differentially regulated by flagellin pretreatment, and SOCS3 expression follows a similar pattern with IL-24. However, their functions in *PA* keratitis are still unknown. We propose that early induction of negative regulator SOCS3 by IL-24 would impair the innate defense against *PA* infection. At the early stage of infection, pathogens induce the expression of AMPs and chemokines, which would recruit immune cells, mainly neutrophils in the case of *PA* infection, to the infection sites. Both AMPs and neutrophils are critical to clear pathogens. Early induction of the negative regulator may hamper the innate defense and hence to promote *PA* keratitis. SOCS3 negatively regulates STAT family proteins, including STAT1 and STAT3. Besides IL-20R cytokines, a number of other cytokines and growth factors signal through the STAT pathway, such as IL-6, IFNs, and G-CSF, etc. IL-6 is an important inflammatory cytokine, and G-CSF stimulates neutrophil production. Therefore, loss of SOCS3 may cause STAT1/3 activation without restriction, resulting in more inflammation in the cornea.

In this study, we first used siRNA approach to knock down IL-24 gene expression to evaluate its function. Our results show that silencing of IL-24 reduces *PA* keratitis with less bacterial burden and less neutrophil infiltration in the cornea. The less keratitis in the silencing group may be due to the early induction of antimicrobial peptides S100A8 and A9 in corneal epithelial cells to kill bacteria directly, resulting in less bacteria enter the cornea to cause pathology. At a later stage of infection (1 dpi), silencing of IL-24 dampens the expression of SOCS3, inflammatory cytokines (including

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IL-6, MMP13, IL-17A, and IL-1β), and anti-inflammatory cytokines (including IL-1RN, S100A8 and A9, and CRAMP). Blockage of IL-20R2 with neutralizing antibody further confirms that down-regulation of IL-24 signaling pathway improves the outcome of *PA* keratitis. In addition, recombinant IL-24 was used to evaluate the gain of function of IL-24 in *PA* keratitis. Recombinant IL-24 leads to more severe keratitis, more live bacteria and neutrophil infiltration in the cornea. Administration of recombinant IL-24 before *PA* infection further increases the expression of SOCS3, pro- and anti-inflammatory cytokines comparing to infection alone. Their up-regulation may be due to the more severe keratitis with recombinant IL-24 application. We also evaluated the function of the downstream mediator, SOCS3, in *PA* keratitis. Silencing of SOCS3 increases the severity of keratitis, bacterial number, and inflammation in cornea, indicating SOCS3 plays an important role in restricting inflammation and infection. Overall, we demonstrate that IL-24 plays a detrimental role in *PA* keratitis, possible due to the early induction of the negative regulator, SOCS3.

Introduction

Increased expression of IL-24 and SOCS3 has been found in many inflamed tissues of various diseases, such as skin inflammation (e.g. psoriasis), rheumatoid arthritis, and inflammatory bowel disease (IBD), etc.(Andoh et al., 2009; Kragstrup et al., 2008; Sa et al., 2007; Uto-Konomi et al., 2012). It is reported that IL-24, signaling through JAK/STAT3 and MAPK pathways, promotes the secretion of pro-inflammatory cytokines IL-8 and MMP-1 in human epidermal keratinocytes under both endogenous and environmental toxic stress (Jin, Choi, Chun, & Noh, 2014). Contrasting to the pro-inflammatory role in skin inflammation and rheumatoid arthritis, IL-24 exhibits repressive

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and regulatory role in IBD (Andoh et al., 2009). In terms of infection, IL-24 has been documented to have protective effects against intracellular pathogens, such as *Salmonella typhimurium* and *Mycobacterium tuberculosis* (Ma et al., 2009; Ma et al., 2011). However, Myles and colleges demonstrated that signaling through IL-20R2 pathway promotes cutaneous infection with MRSA through the inhibition of IL-1β and IL-17A production. Yet they did not directly evaluate the functions of IL-24 (Myles et al., 2013).

Both cytokines and pathogen components can stimulate SOCS3 gene expression (Carow and Rottenberg, 2014). Studies in different mouse models have proven the critical importance of SOCS3 in restraining inflammation and promoting optimal levels of protective immune response against the infection (Rottenberg and Carow, 2014). Enhanced SOCS3 expression has been reported to convey resistant to *M. tuberculosis* infection (Carow et al., 2013; Carow and Rottenberg, 2014). On the other hand, some pathogens have evolved to modify host SOCS3 expression to evade the immune response. For example, *Epstein-Barr* virus and *Herpes simplex* virus can stimulate SOCS3 expression to suppress type I IFN production, thereby subverting the host immune response (Michaud et al., 2010; Yokota et al., 2005). Demirel *et al*. found that SOCS3, but not SOCS1, was induced in human bladder epithelial cells when the cells challenged with uropathogenic *E. coli*. They believed that *E. coli* was able to modify SOCS3 and STAT3 signaling in human bladder epithelial cells to circumvent the host response and cause urinary tract infection (Demirel, Save, Kruse, & Persson, 2013).

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To date, the functions of IL-24 and SOCS3 in bacterial keratitis and their regulatory relationship have not been explored. We used IL-24 siRNA and IL-20R2 neutralizing antibody to evaluate the loss of function of IL-24 signaling pathway in *PA* keratitis. In addition, mouse recombinant IL-24 was used to evaluate the gain of function of IL-24. siRNA approach was also used to evaluate the important role of SOCS3 in *PA* keratitis. Our studies demonstrate that IL-24 signaling pathway increases the susceptibility of mouse cornea to *PA* infection. *PA* induces early expression of IL-24, which readily induces SOCS3 expression, resulting in suppression of the necessary immune response to combat infection at an early time point.

Results

Silencing of IL-24 improves the outcome of *PA* keratitis in mouse cornea

To determine whether IL-24 influences the host defense against *PA* keratitis, we subconjunctivally injected B6 mice with IL-24 siRNA, with non-specific siRNA as the control. Western blotting analysis of the whole corneas at 1 dpi revealed an effective knockdown of IL-24 mRNA by siRNA, as well as the downregulation of STAT3 activation (Fig. 21A and B).

The progress of *PA* keratitis in the control siRNA and IL-24 siRNA treated corneas was monitored for 3 days. Micrographs taken daily showed that knocking down IL-24 attenuated the severity of keratitis, compared to the control group, at all three days examined (Fig. 21C). At 3 dpi, the control corneas developed severe keratitis, thick opacity covering the whole cornea, and surface irregularity; while the corneas injected with IL-24 siRNA had moderate keratitis with lighter opacity located at the center of the cornea (Fig. 21C). The severity of keratitis was quantitated with a 12-scale clinical score

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system by evaluating the area and density of opacity and surface irregularity (Wu et al., 2003). The clinical scores assigned to IL-24 silencing mice were significantly lower than those of their control counterparts (Fig. 21D). Mice were sacrificed at 3dpi and whole corneas were subjected to bacterial counting and MPO measurement to evaluate neutrophil infiltration. Silencing of IL-24 resulted in lower bacterial burden and reduced influx of neutrophils compared to the control corneas (Fig. 21E and F).

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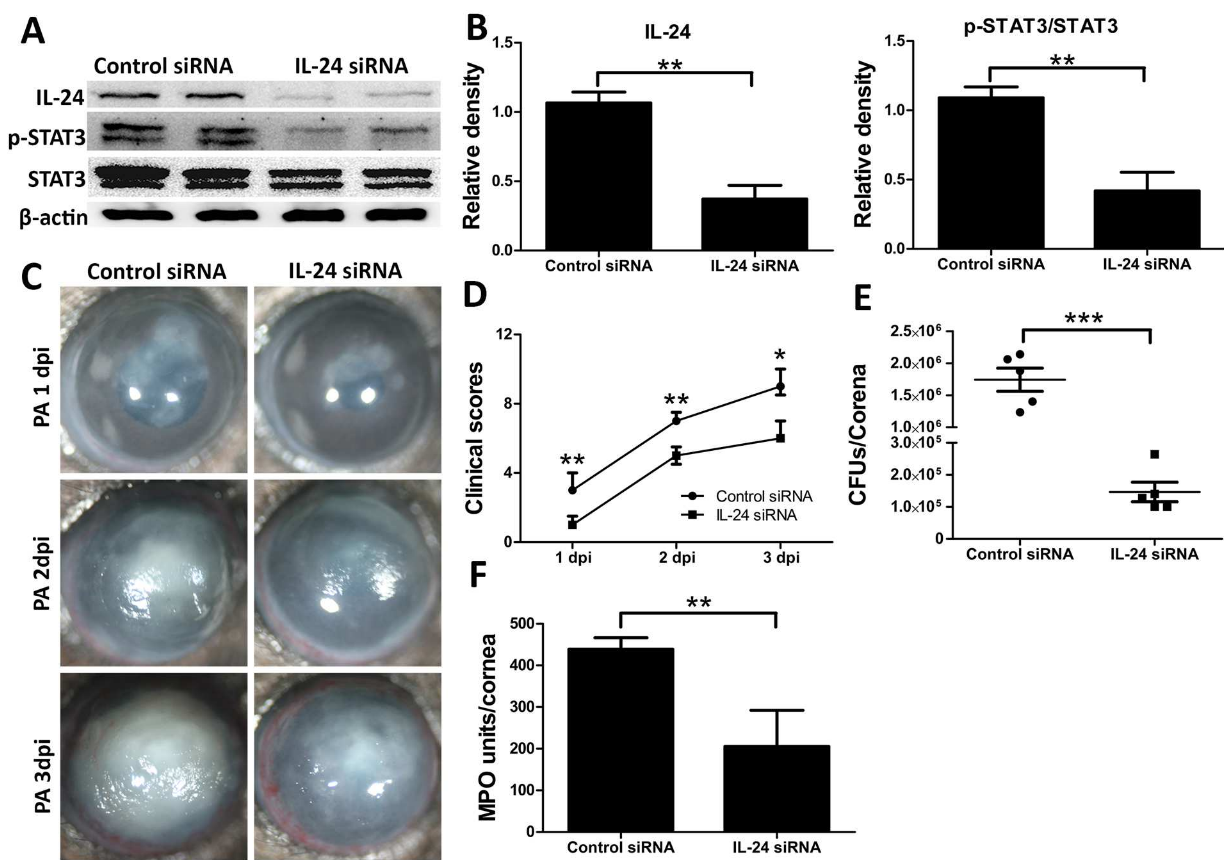

Figure 21 Silencing of IL-24 attenuates the severity of *PA* infection in mouse cornea. Mice were subconjunctivally injected with IL-24 siRNA (10μM/μl, 5ul) twice in two days. Corneas were inoculated with *PA* 6h after the second injection. Non-specific siRNA serves as control. (A) Immunoblot analysis of IL-24, p-STAT3, and STAT3 in cell lysates of IL-24 siRNA- or control siRNA-treated corneas at 1 dpi. β-actin serves as the loading control. (B) Quantification of protein levels based on the densitometry of the Western blots in A. (C) Mice were monitored and photographed daily up to 3 dpi. (D) Clinical scores were assigned to each cornea daily and plotted as median + interquartile range. At 3 dpi, the corneas were excised and subjected to bacterial plate counting (E) and MPO assay (F). P values were generated by Man-Whitney test (D) or unpaired student t test (B, E and F). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Data are representative of three independent experiments with five mice per group (B, E and F: mean + s.e.m.).

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Since there was basal expression of IL-24 detected at the protein level, we treated the corneas with IL-24 siRNA and assessed its effects on the expression of several antimicrobial peptides (AMPs) and cytokines at 6 hpi in mouse CECs. As shown in Fig. 22A, without the infection, the expression of the AMP S100A8 increased 3 folds ($P<0.05$, ANOVA) after IL-24 knockdown. *PA* infection induced the expression of both S100A8 and S100A9, yet silencing of IL-24 further elevated their expression in the infected corneas. The expression of CXCL10 was also induced by *PA* infection, and further increased when silencing of IL-24. The expression of SOCS3 was upregulated after *PA* infection. IL-24 knockdown resulted in the downregulation of SOCS3 expression. The expression of inflammatory cytokines MMP13, IL-1β, and its natural inhibitor, IL-1RN, was induced by *PA* infection. IL-24 knockdown dampened their expression to a state similar to the control siRNA without infection in mouse CECs. No significant expression change of Cathelicidin-related antimicrobial peptide (CRAMP) or IL-6 was detected at 6 hpi. The expression of calprotectin and CXCL10 was further confirmed in protein level (Fig. 22B). Basal expression of both calprotectin and CXCL10 was detected in the uninfected corneas. Consistent with the mRNA expression, the protein expression of calprotectin and CXCL10 was induced by PA infection, and further enhanced when knocking down of IL-24.

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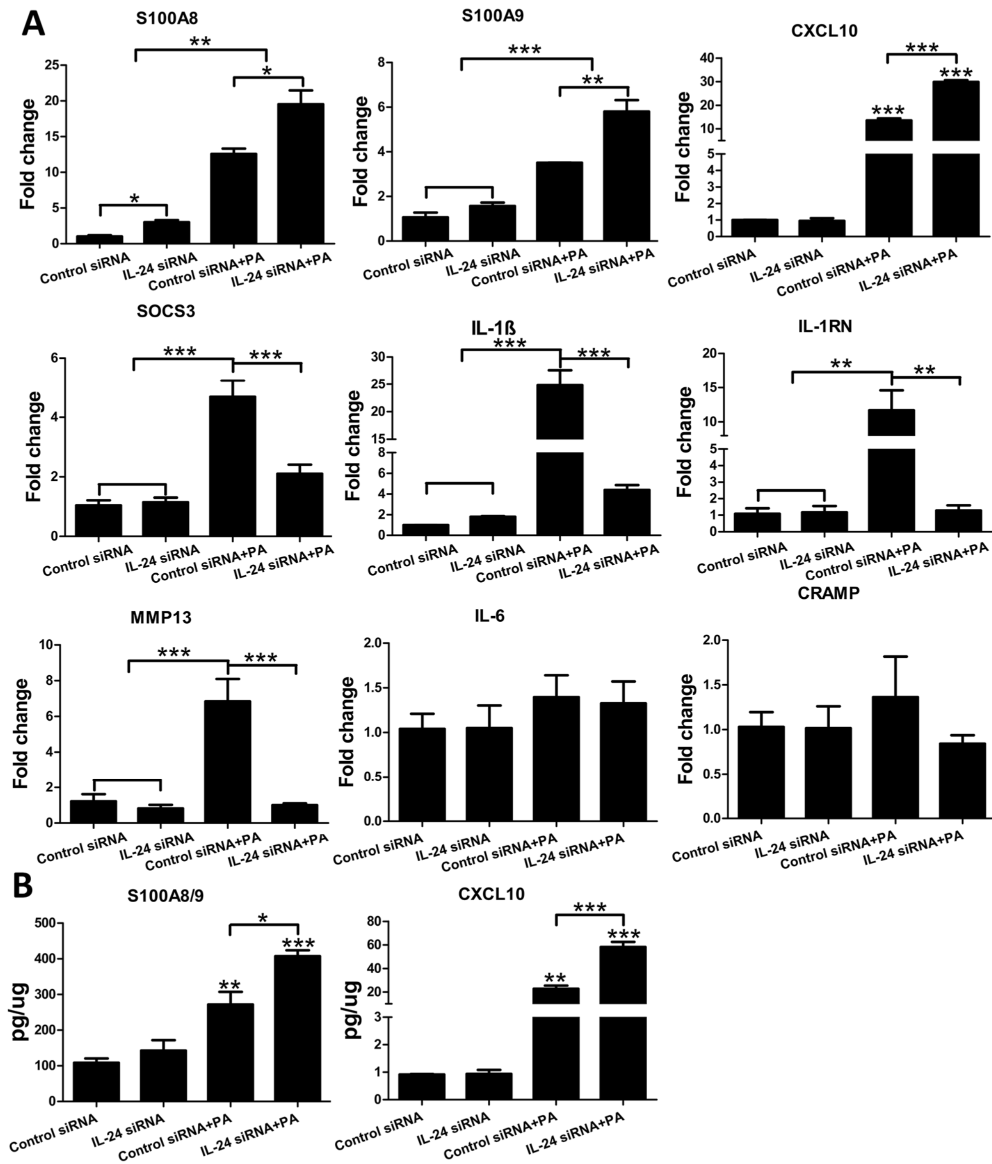

Figure 22 Silencing of IL-24 upregulates the expression of antimicrobial peptides and chemokine in mouse corneal epithelial cells in response to *PA* infection. Mouse corneas were treated with IL-24 siRNA or control siRNA and inoculated with *PA* as in figure 3. Corneal epithelial samples were collected at 6hpi for qPCR (A) and ELISA (B) analysis. For q-PCR, results are presented relative to those of control siRNA treated, uninfected corneas, set as 1. *P < 0.05, **P < 0.01, and ***P < 0.001 (ANOVA). Data are representative of three independent experiments with at least four corneas per group (mean + s.e.m.).

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As an early responsive cytokine, IL-24 may induce the expression of other cytokines and/or AMPs in infected corneas. To that end, we used real-time PCR to assess the expression of eight different genes at 1dpi in the corneas (Fig. 23A). While most genes were unaffected by the downregulation of IL-24, the expressions of IL-1RN (7.2 fold increase, $p<0.05$), S100A9 (3.3 fold increase, $p<0.05$), and IL-17A (0.6 fold decrease $p>0.05$) were altered by IL-24 silencing. Infection induced marked upregulation of SOCS3, IL-6, MMP13, IL-17A, IL-1β and its natural inhibitor IL-1RN, AMP S100A8 and A9, and CRAMP at 1dpi. Knocking down IL-24 resulted in the downregulation of the following genes: SOCS3 (1.47 fold), IL-6 (4), MMP13 (1.73), IL-17A (3.3), IL-1β (3.0), IL-1RN (5.72), S100A8 (1.98) and A9 (1.45), CXCL10 (2.0), and CRAMP (4.2 fold) (Fig. 23A). In the IL-20 subfamily, infection increased the expression of IL-19, but not IL-20 or IL-22, at 18hpi. Silencing of IL-24 significantly dampened IL-19 expression (Fig. 23A). The expression of IL-6, IL-1β, IL-1RA, and heterodimer S100A8/9 was confirmed at the protein level by ELISA analysis (Fig. 23B). While the basal levels of IL-6 and IL-1β were undetected (in physiological condition, IL-6 concentration in human serum is 1-5 pg/ml (Hunter and Jones, 2015)), basal expression of IL-1RA and S100A8/9 was observed in the control and IL-24 siRNA treated, non-infected corneas. As shown at the mRNA level, IL-6, IL-1β, IL-1RA, and S100A8/9 were elevated at the protein level by *PA* infection and significantly attenuated by IL-24 silencing. The expression of SOCS3 was further confirmed in protein level. Silencing of IL-24 downregulated SOCS3 protein expression at 1 dpi (Fig. 23C). The down-regulation of the cytokines and AMPs after IL-24 silencing at 1dpi in the cornea may be related to the diminished keratitis in the silencing group.

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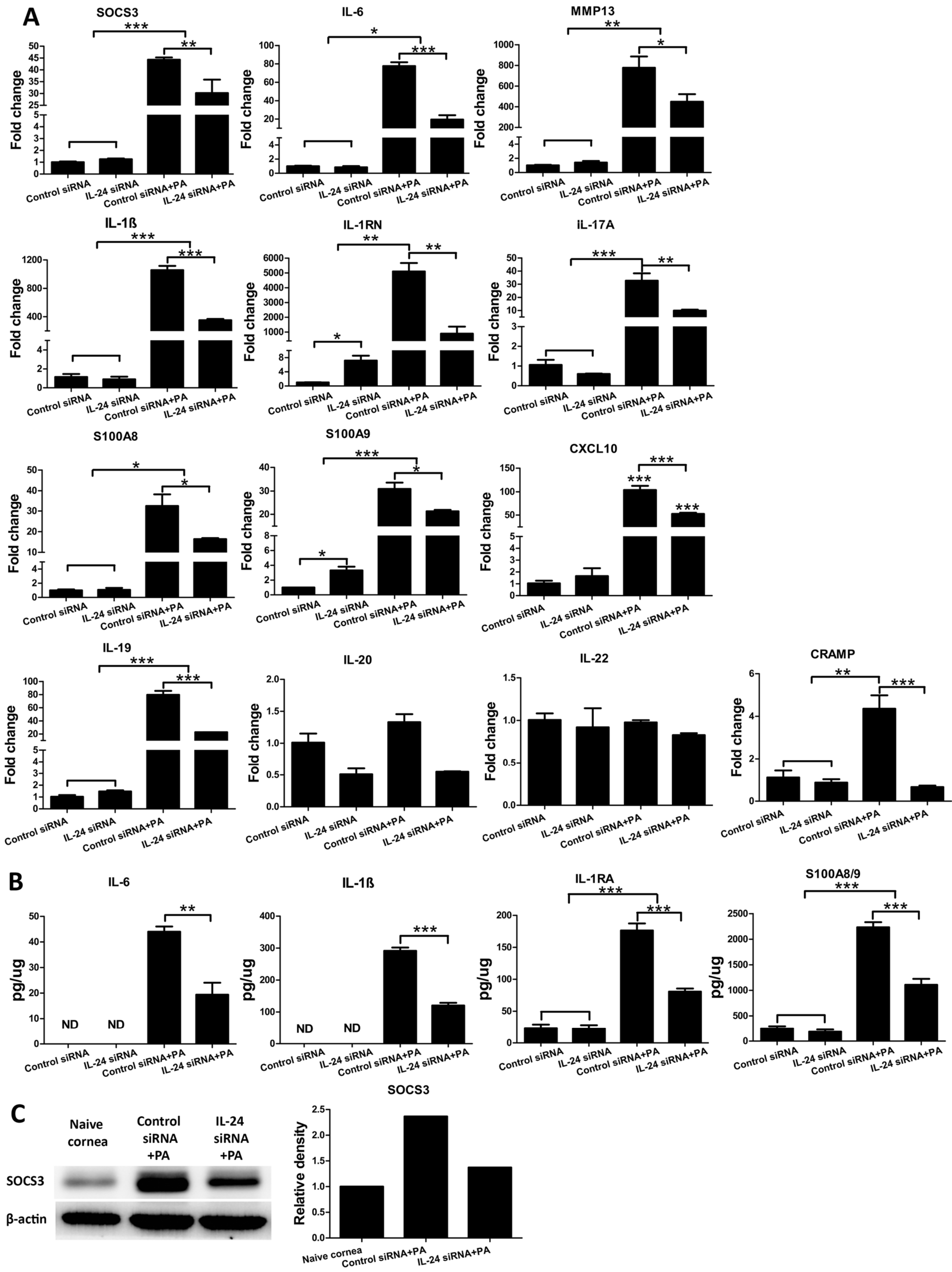

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Figure 23 Silencing of IL-24 dampens cytokine expression in mouse cornea in response to *PA* infection. Mouse corneas were treated with IL-24 siRNA or control siRNA and inoculated with *PA* as in figure 3. (A) Whole cornea samples were collected at 1dpi for qPCR analysis. Results are presented relative to those of control siRNA treated, uninfected corneas, set as 1. Cell lysates from whole corneal samples at 1dpi were subjected to ELISA (B) and Western Blot (C) analysis. (C) Blots were quantified based on the densitometry. *P < 0.05, **P < 0.01, and ***P < 0.001 (ANOVA or student T test). Data are representative of three independent experiments with at least four corneas per group (mean + s.e.m.) (A and B). Experiment was performed once. (C) ND: not detected.

Blockage of IL-20R2 decreases the severity of *PA* keratitis in mouse cornea

Next, we assessed the effects of blocking IL-20R2, the common receptor chain for IL-19, IL-20, and IL-24, to further verify the effect of the IL-24 signaling pathway on *PA* keratitis (Fig. 24). While the IgG control corneas developed keratitis gradually within 3 days, the anti-IL-20R2 antibody-treated corneas had slight corneal cloudiness at day 1, which progressed to cloudy corneas at day 2, and surprisingly resolved to cloudiness with the outline of the iris and pupil discernable at day 3 (Fig. 24A). Clinical scoring revealed that the anti-IL-20R2 group started with a lower score at day 1, increased the score with a slope similar to that observed in the IgG control group at day 2, with scores significantly lower than the control IgG treated ones at day 3 (Fig. 24B). The blockage of IL-20R2 reduced the bacterial burden and MPO units in the corneas at 3 dpi as well (Fig. 24C and D).

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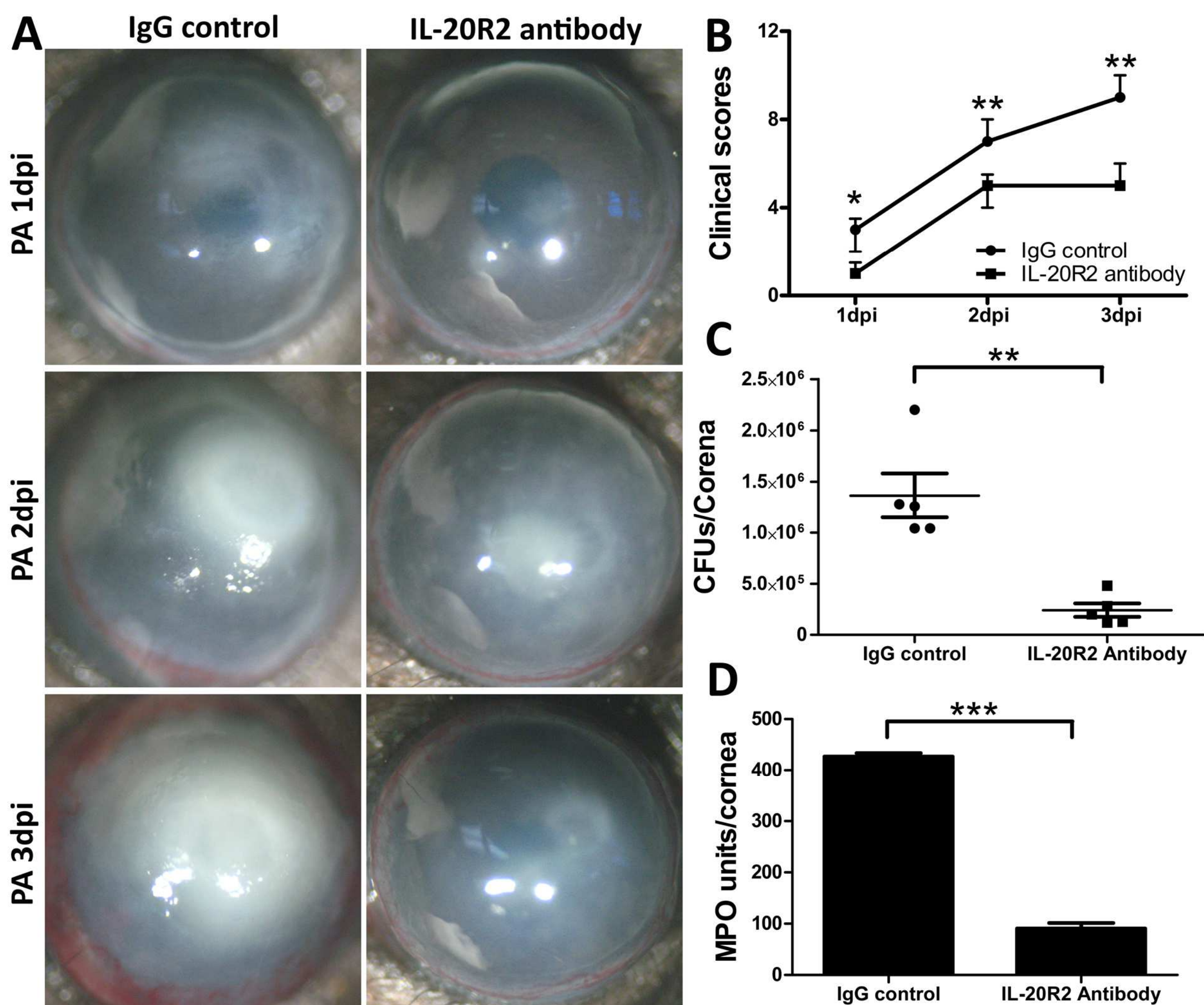

Figure 24 IL-20R2 neutralizing antibody decreases the severity of *PA* keratitis in mouse cornea. Mice were subconjunctivally injected with IL-20R2 neutralizing antibody (200ng/5ul) 4h before the inoculation with *PA*. Mouse IgG serves as control. (A) Mice were monitored and photographed daily up to 3 dpi. (B) Clinical scores were assigned to each cornea daily and plotted as median + interquartile range. At 3 dpi, the corneas were excised and subjected to bacterial plate counting (C) and MPO assay (D). P values were generated by Man-Whitney test (B) or unpaired student t test (C and D). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Data are representative of two independent experiments with five mice per group (D and E: mean + s.e.m.).

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Recombinant IL-24 increases the susceptibility of mouse cornea to *PA* infection

As a complementary approach, we assessed the effects of recombinant IL-24 on the severity of *PA* keratitis. Subconjunctival injection of recombinant mouse IL-24 four hours prior to *PA* inoculation resulted in more severe keratitis than that of the control with 0.1% BSA injection (Fig. 25A). The control corneas were lightly covered (30%) at day 1, 80% covered at day 2, and fully covered at day 3 with opacity. Surface irregularity was also apparent at day 3. In recombinant IL-24-treated corneas, there was a sign of central melting at day 1, central ring with heavy opacity at day 2, and heavy opacity covered the entire cornea with corneal ulceration and neovascularization present at day 3, pathologies usually seen in the control corneas at day 5 (Fig 25A). The clinical scores revealed significantly higher disease severity in the recombinant IL-24-treated group than that observed in the BSA control group (Fig. 25B). A single dose of recombinant IL-24 resulted in significantly higher bacterial burden and neutrophil infiltration than in the BSA control group at 3 dpi (Fig. 25C and D).

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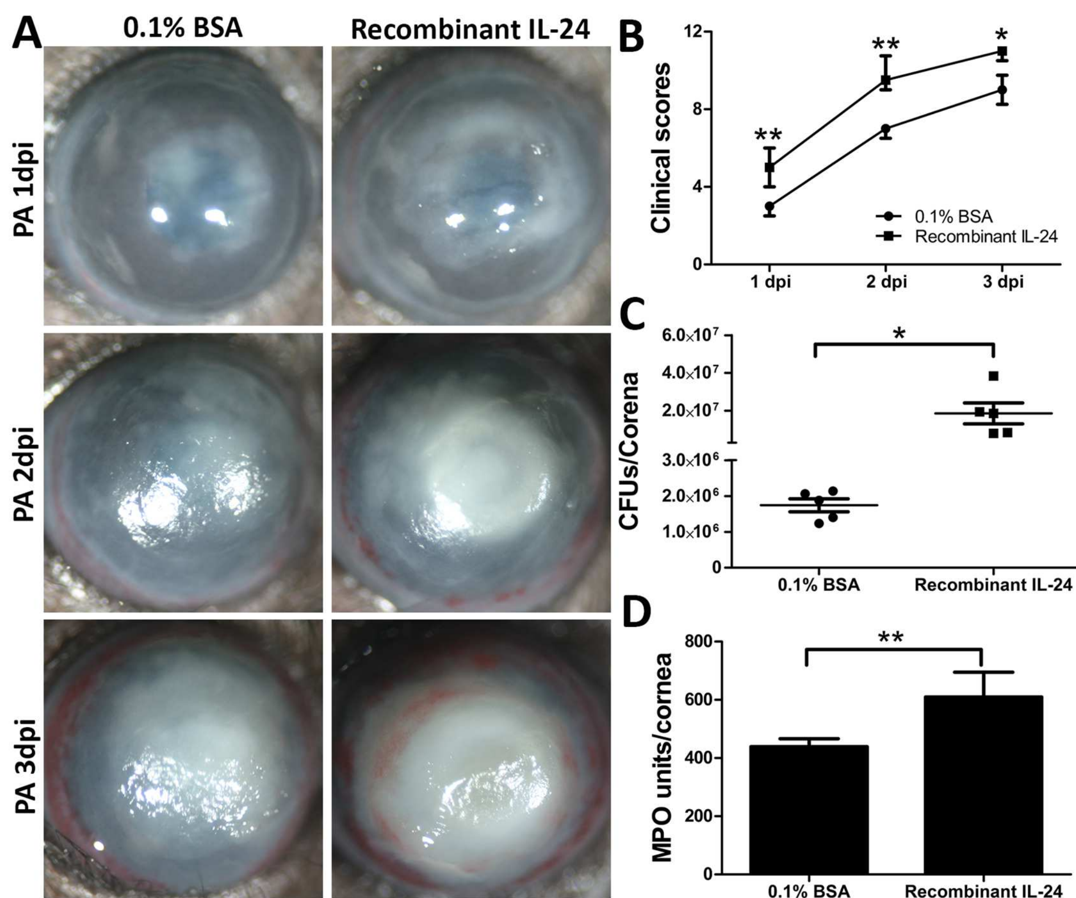

Figure 25 Recombinant IL-24 increases the susceptibility of the mouse cornea to *PA* infection. Mice were subconjunctivally injected with either recombinant mouse IL-24 (200ng in 5ul 0.1% BSA) or 0.1% BSA as control. Mouse corneas were inoculated with *PA* 4 h after the injection of recombinant IL-24. (A) The infected corneas were photographed daily. (B) The severity of keratitis was assessed with clinical scores. At 3 dpi, the corneas were excised and subjected to bacterial load (C) and MPO unit determination (D). P values were generated by Man-Whitney test (B) or unpaired student t test (C and D). $^*P < 0.05$ and $^{**}P < 0.01$. Data are representative of three independent experiments with five mice per group (B: median + interquartile range; C and D: mean + s.e.m.).

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The expression of the same set of genes tested in IL-24 downregulated corneas was also assessed in recombinant IL-24-treated corneas (Fig. 26). In non-infected corneas, recombinant IL-24 triggered significant upregulation of IL-17A (4.8 fold increase, $p<0.01$); while the expression of IL-1RN (2.5 fold decrease, $p<0.01$) and S100A8 (0.5 fold decrease, $p>0.05$) was downregulated, contrasting those of IL-24 siRNA-treated corneas. The presence of recombinant IL-24 significantly augmented the infection-induced expression of these genes, except IL-19. Administration of recombinant IL-24 before *PA* infection did not significantly upregulate IL-19 expression. No significantly elevated expression of IL-20 or IL-22 was detected with or without exogenous IL-24 application before *PA* infection.

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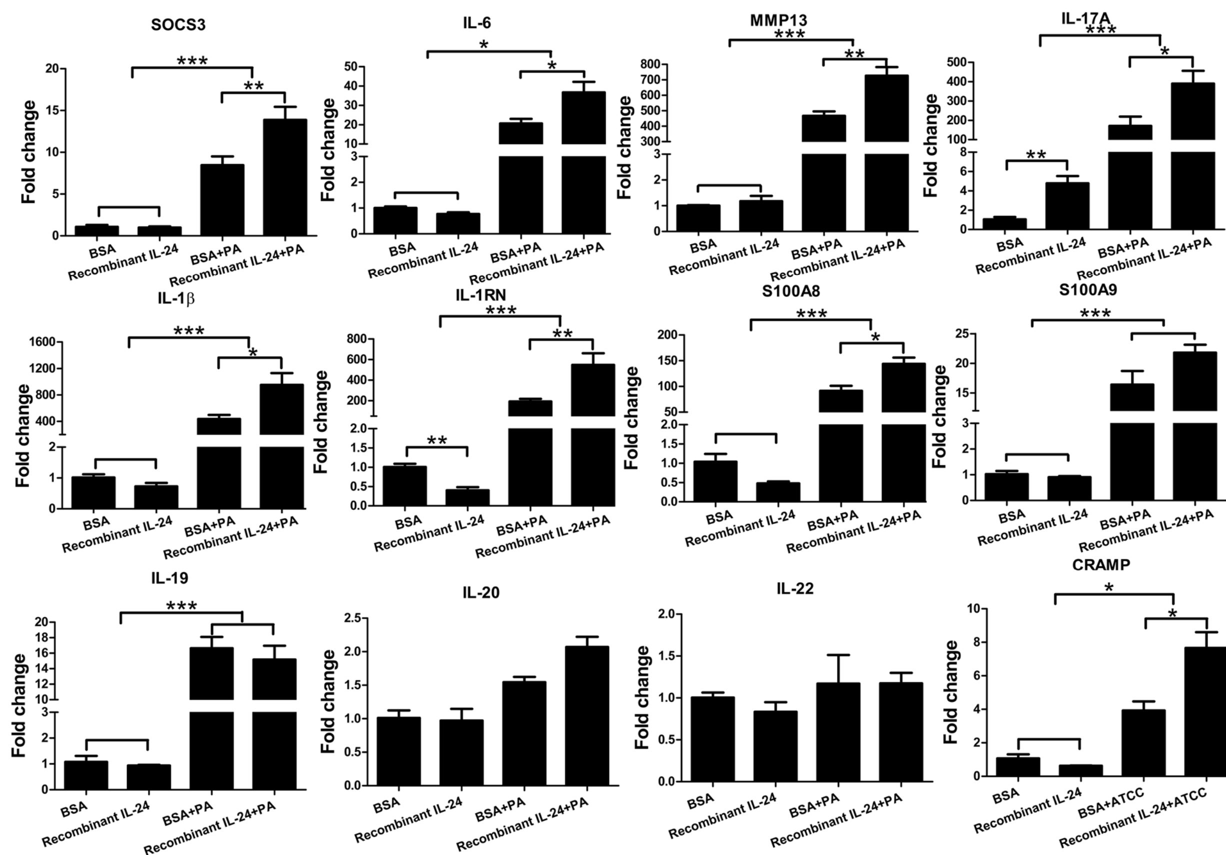

Figure 26 Recombinant IL-24 enhances cytokine expression in mouse cornea in response to *PA* infection. Mouse corneas were treated with recombinant mouse IL-24 or 0.1%BSA and inoculated with *PA* as in figure 6. Whole cornea samples were collected at 1dpi for qPCR analysis. Results are presented relative to those of 0.1% BSA treated, uninfected corneas, set as 1. *P < 0.05, **P < 0.01, and ***P < 0.001 (ANOVA). Data are representative of three independent experiments with at least four corneas per group (mean + s.e.m.).

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Silencing of SOCS3 impairs the control of *PA* infection in mouse cornea

Having shown that SOCS3, an intrinsic negative regulator to the STAT3 pathway, was expressed in infected corneas in an IL-24 related manner, we next investigated the effects of SOCS3 on the host response to *PA* infection in B6 mouse corneas using a siRNA approach. Western blotting demonstrated the suppression of infection-induced SOCS3 expression by SOCS3 siRNA at the protein level at 1 dpi (Fig. 27A). Both p-STAT3 and STAT3 levels were upregulated when silencing of SOCS3 (Fig. 27B). Downregulation of SOCS3 resulted in an increase in the susceptibility of mouse corneas to *PA* infection, compared to the control siRNA-treated corneas (Fig. 27C). Clinical scores assigned to the SOCS3 silencing group were significantly higher than those observed in the control group (Fig. 27D). Mice were sacrificed at 3 dpi to assess bacterial burden and MPO units in the cornea. Silencing of SOCS3 resulted in significantly higher bacterial burden and more infiltrated neutrophils in mouse corneas than the control (Fig. 27E and F).

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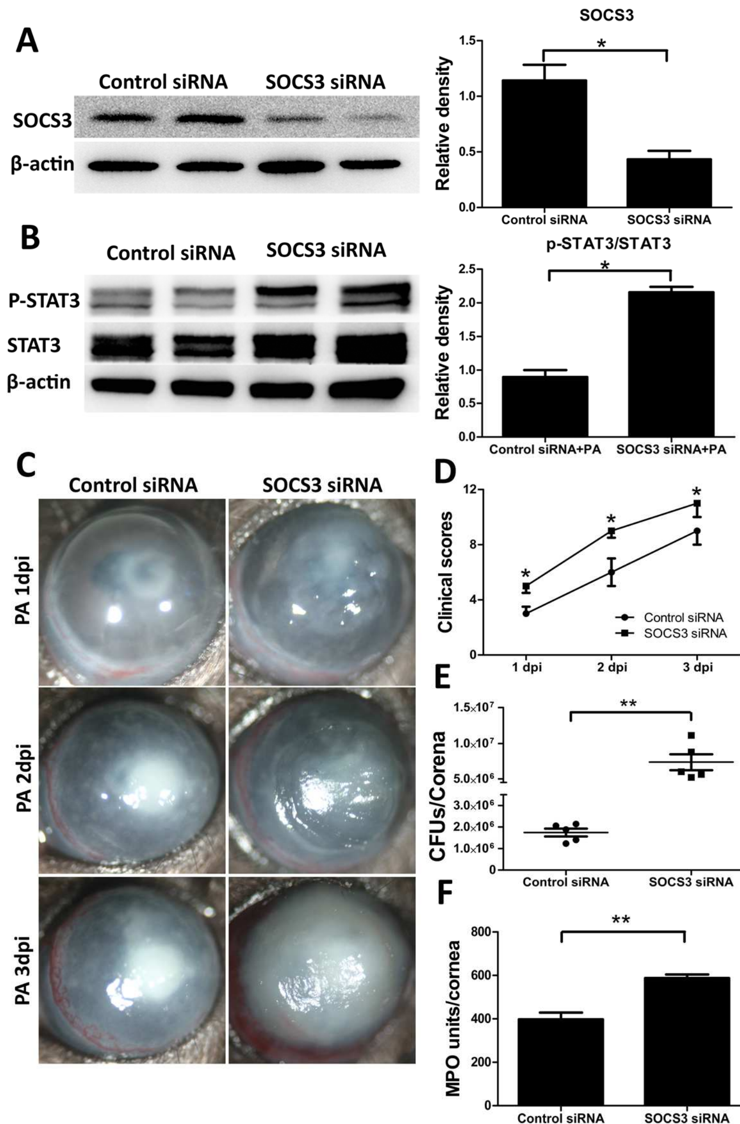

Figure 27 Silencing of SOCS3 renders mouse corneas more susceptible to *PA* infection. Mice were subconjunctivally injected with SOCS3 siRNA or control siRNA, followed by *PA* inoculation as in Fig. 3. (A) Immunoblot analysis of SOCS3 in cell lysates of the whole cornea at 1dpi to ensure knockdown efficiency. (B) Immunoblot analysis of p-STAT3 and STAT3 in cell lysates of the whole cornea at 1dpi. β-actin was used as a loading control. Blots were quantified based on the densitometry. (C) The infected corneas were photographed daily. (D) The severity of keratitis was quantitated with clinical scores. Bacterial load (E) and neutrophil infiltration (F) were measured at 3 dpi. *P < 0.05, **P < 0.01 and ****P* < 0.001 (Man-Whitney test or unpaired student t test). Data are representative of three independent experiments with five mice per group (D: median + interquartile rang; B, E and F: mean + s.e.m.) (A, C-E). Experiment was performed once (B).

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Discussion

In this part of the study, we demonstrated that silencing of IL-24 with siRNA alleviated the severity of *PA* infection and recombinant IL-24 protein aggravated the infection. The expression of AMP S100A8 and A9 was increased in epithelial and whole corneal samples by silencing of IL-24 alone, whereas recombinant IL-24 had opposite effect. *PA* infection induced S100A8 and A9 expression; however, their expression further increased in the epithelia at the early time point (6 h) post infection when IL-24 was silenced. These data demonstrate that silencing of IL-24 may directly or indirectly regulate the expression of S100A8 and A9, which may kill bacteria directly and account for the reduced keratitis seen at the later time points of infection. As the infection and inflammation progress, cytokine expression may be more related to the severity of keratitis, as demonstrated by the subsidence of the cytokines and antimicrobial peptides after IL-24 silencing at 1dpi, as less keratitis developed in the silencing group; conversely, their expression levels at the same time point further elevated with exogenous IL-24 administration prior to the infection.

PA is still mainly located in the corneal epithelial layer at 6 hpi. At 1 dpi, *PA* invades into corneal stroma and causes majority of epithelial cell death. The major cell type in the cornea is infiltrating cells. Therefore, the different expression of S100A8 and A9 may be cell type specific between corneal epithelial cells at 6 hpi and whole corneas at 1 dpi when silencing of IL-24.

CXCL10 is also known as interferon-γ-inducible protein 10 (IP-10). It signals through the receptor CXCR3 to recruit immune cells, such as natural killer cells and plasmacytoid dendritic cells to sites and infection and inflammation (Cao and Liu, 2007;

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Cole et al., 2001; Mohan et al., 2005). Besides functioning as a chemokine, CXCL10 possesses direct antimicrobial effects against pathogens (Cole et al., 2001; Yoon et al., 2013). Our lab has demonstrated the important functions of interferons and CXCL10 in immunity and infection, even in wound healing (Liu et al., 2014; Yan et al., 2016; Yoon et al., 2013). By signaling through their receptors, interferon activates STAT1/3 to regulate CXCL10 expression (Ivashkiv and Donlin, 2014). We observed *PA* infection induces CXCL10 expression in mouse CECs at 6hpi; silencing of IL-24 further augments its expression. Silencing of IL-24 induces less expression of the negative regulator SOCS3. Without the negative regulator, interferon pathway is activated without restriction to further induce CXCL10 expression. The increased CXCL10 in the cornea may kill bacteria directly, contributing to less keratitis and less bacterial numbers seen at a later time point in the IL-24 silencing group.

The effect of neutralizing antibody that neutralizes IL-20R2, the common receptor chain in IL-24 signaling pathway, further confirmed the detrimental role of IL-24/IL-20R cytokines in *PA* keratitis. The correlated expression of IL-24 and SOCS3 indicates that the detrimental effects of IL-24 may be ascribed to its expedient induction of the expression of the negative regulator, SOCS3. The ability of IL-24 to induce the expression of the negative regulator is in line with the anti-inflammatory property of the IL-10 family cytokines in general (Jones and Flavell, 2005). Indeed, the regulatory cascade, IL-24/STAT3/SOCS3, is also reported to contribute to the suppression of mucosal inflammation in inflammatory bowel disease (Andoh et al., 2009). In addition, it is reported that the anti-inflammatory properties of IL-20R cytokines actually promotes cutaneous infection with MRSA in an IL-1β and IL-17 related manner (Myles et al.,

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2013). Our study further demonstrated that IL-24 possesses immunosuppressive activity that may diminish the host's ability to control infection with a different mechanism: the robust induction of SOCS3 expression at early stages of infection.

SOCS3 is an important negative regulator to maintain or restore tissue homeostasis and is involved in a variety of diseases. Keratinocyte-specific SOCS3 conditional knockout mice developed severe skin inflammation with epidermal hyperplasia (Uto-Konomi et al., 2012). It is reported that SOCS3 expression levels positively correlate with the severity of inflammation, increasing when the diseases progress and declining when the diseases subside (Chaves de Souza et al., 2013; Maier et al., 2002). Our study also shows SOCS3 transcripts parallel with the severity of keratitis when the mouse corneas were treated with IL-24 siRNA or recombinant IL-24 prior to infection. Recombinant SOCS3 constructed with cell penetrating signal has been shown to inhibit inflammation and apoptosis, and protect mice from lethal doses of enterotoxin B (SEB) or LPS (Jo et al., 2005). The fact that silencing of SOCS3 increases the severity of keratitis with greater bacterial burden in mouse corneas indicates that SOCS3 not only hampers excessive inflammation, but also shapes the corneal response to pathogens. Our results are consistent with previous reports showing that myeloid or lymphoid cell specific SOCS3 knockout rendered mice more susceptible to *M. tuberculosis* infection (Carow et al., 2013).

Silencing of SOCS3 increases the severity of *PA* keratitis. SOCS3 is the main negative regulator of IL-6/gp130 signaling pathway, and is a direct target gene of STAT3 (Garbers et al., 2015). IL-6 is a pleiotropic cytokine. It is reported that IL-6 can utilize two mechanisms to mediate its biological functions: classical signaling and trans-

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signaling (Hunter and Jones, 2015). Classical signaling refers to a process in which IL-6 binds to the receptor complex consisting of membrane-bound IL-6Rα (mIL-6Rα) and gp130 to mediate its biological effects. Since mIL-6Rα is only expressed in certain cell types, classical IL-6 signaling occurs in a restricted numbers of cells such as certain leukocyte subsets (Jones et al., 2011). Trans-signaling denotes that IL-6 binds to a soluble IL-6Rα protein (sIL-6R) to form an IL-6/sIL-6R complex, which would subsequently bind to gp-130 receptor unit in plasmid membrane to induce signal transduction. Gp130 is ubiquitously expressed; thus IL-6 trans-signaling occurs in a broad spectrum of cells (Peters et al., 1996; Rose-John and Heinrich, 1994). It is believed that while the classical signaling mediates the anti-inflammatory and regenerative effects of IL-6, IL-6 trans-signaling accounts predominantly for the pro-inflammatory activities of IL-6 (Scheller et al., 2011). It is reported that selective inhibition of IL-6 trans-signaling may become a potential intervention to control inflammatory arthritis (Nowell et al., 2009). In response to infection, both the levels of IL-6 and sIL-6R are increased to mount an inflammatory response (Honda et al., 1992; Jones et al., 2001; Novick et al., 1989). SOCS3 negatively regulates the IL-6/sIL-6R signaling pathway to prevent destructive events. Silencing of SOCS3 renders the signal to be transduced without constriction, therefore causing more severe *PA* keratitis in a mouse model. Gp130 mutant mice generated by knock-in mutation of IL6st lose the binding ability of the mutant gp130 to SOCS3. These mice display sustained activation of STAT1 and STAT3 and develop unrestrained inflammation (Atsumi et al., 2002).

SOCS3 not only negatively regulates IL-6 trans-signaling pathway, but also is induced by STAT3 to restrain neutrophil production and limit inflammation (Fielding et

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al., 2008; Lee et al., 2002). Silencing of SOCS3 may facilitate the G-CSF signal transduction in bone marrow granulocytic progenitor cells to increase neutrophil production, thus causing sustained inflammation. In addition, by enhancing STAT3 activation, IL-6 cooperates with G-CSF to induce neutrophil production (Yan et al., 2013). Therefore, mouse cornea developed more severe keratitis with more PMNs infiltration when silencing of SOCS3.

In summary, our study indicates a detrimental role of IL-24 in corneal innate immunity against *PA* infection: while down-regulation of IL-24 protected mouse corneas from *PA* infection, exogenous IL-24 worsened *PA* keratitis. SOCS3, as a downstream effector of IL-24, plays important roles in restricting the inflammation and infection, as demonstrated by increased inflammation and more bacterial burden when silencing of SOCS3. However, early induction of the negative regulator SOCS3 by IL-24 may account for the detrimental effects of IL-24 in *PA* keratitis.

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CHAPTER 3: EXPLORE THE REGULATORY RELATIONSHIP OF IL-24 AND SOCS3 *IN VITRO* IN PRIMARY HUMAN CORNEAL EPITHELIAL CELLS (HCECS).

Summary

We have shown the expression patterns and functions of IL-24 and SOCS3 in a mouse model of *PA* keratitis. Our next question is whether these results can be translated into human, since our ultimate goal is to treat human diseases. We propose that *PA* challenge induces IL-24 expression in HCECs, and IL-24 regulates SOCS3 expression. It is reported that human keratinocytes express IL-20R cytokines when challenged with MRSA or treated with IL-1β (Myles et al., 2013). Therefore, IL-24 expression may be triggered by *PA*, and further induced by IL-1β as the infection and inflammation continue. The induction of negative regulator by IL-24 would be consistent with its anti-inflammatory property as a member in the IL-10 family.

The importance of corneal epithelial cells has been demonstrated in innate immune defense against pathogen invasion. Primary human corneal epithelial cells were used to evaluate whether IL-20R cytokines can be induced when challenged with heat-killed *PA*. Consistent with the mouse model, *PA* challenge induced early expression of IL-24 and late expression of IL-19 in primary HCECs: IL-24 expression gradually increased during the course of *PA* challenge, while the up-regulation of IL-19 was only detected at 4 h post challenge. In contrast to the mouse model, heat-killed *PA* challenge also induced IL-20 expression in primary HCECs, with peak expression at 2 h and a subsidence at 4 h post challenge. Therefore, heat-killed *PA* can induce the expression of IL-20R cytokines in primary HCECs. When primary HCECs were treated with human recombinant protein, recombinant IL-24 can readily induce SOCS3 expression, whereas the same amount of recombinant IL-20 failed to do so. These

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results indicate that the regulatory relationship of IL-24 and SOCS3 identified in the mouse model is relevant to human. The increased IL-20 expression in primary HCECs may mediate other effects in response to *PA* challenge. IL-1β, as a master regulator of immune response, promotes the expression of IL-20R cytokines. However, both IL-24 and IL-20 cannot induce the expression of inflammatory cytokine IL-1β or IL-6 in primary HCECs, further confirming their immune regulatory role as members in the IL-10 super family.

Introduction

IL-20R cytokines share identical receptors, especially for IL-24 and IL-20, indicating their functions may be interchangeable between species. It is reported that during mouse skin infection with MRSA, both IL-19 and IL-24 are induced during the initial hours of infection, with more abundance of IL-19 than IL-24 in the later stage of infection; no elevated of IL-20 was detected. However, primary human keratinocytes produced more IL-20 than either IL-19 or IL-24 in the initial hour when challenge with MRSA. Thus, the author concluded that human IL-20 might mirror the effects of IL-19 seen in mice during cutaneous infection (Myles et al., 2013). The expression patterns of IL-20R cytokines in primary HCECs in response to *PA* are still unknown.

IL-1β, as an early responsive gene and a master regulator of innate immunity, may induce other cytokine expression in *PA* keratitis. It is reported that IL-1β can induce the expression of all three IL-20R cytokines in keratinocytes, with IL-19 being the most prominent one (1000 folds comparing to 10 folds of IL-20 or IL-24) (Kunz et al., 2006). In addition, IL-1β can activate the transcription factors AP-1 and C/EBP-β to promote IL-24 expression in human isolated colonic subepithelial myofibroblasts (Andoh et al.,

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2009). In contrast, Myles reported IL-20R cytokines can promote cutaneous infection with MRSA through the suppression of IL-1β (Myles et al., 2013). The regulatory relationship of IL-1β and IL-24 in corneal epithelial cells, however, remains unknown.

In this study, by using *in vitro* cell culture system, we demonstrated that heat-killed *PA* challenge induced IL-20R cytokine expression in primary HCECs. While IL-1β promoted IL-20R cytokine expression, IL-24 only induced robust SOCS3 expression, but not inflammatory cytokine expression in HCECs.

Results

Heat-killed *PA* induces IL-20R cytokine expression in primary HCECs

To determine whether HCECs express IL-20R cytokines in response to bacterial challenge, primary HCECs were treated with heat-killed *PA* (MOI=50). Cells were collected at various time points for PCR analysis to evaluate related cytokine expression (Fig. 28). Heat-killed *PA* stimulated progressive upregulation of IL-24 in cultured HCECs. The expression of IL-20 increased gradually at the first 2 hours, but subsided to a level equivalent to control at hour 4. SOCS3 expression significantly elevated during the first 2 hours of stimulation and subsided to control level at hour 4 as well. Consistent with the results obtained from mouse CECs, IL-19 was not induced until a later time point, at hour 4. As to the inflammatory cytokines, heat-killed *PA* induced marked elevation of IL-6 and IL-1β at hour 1 and subsided, but remained significantly higher than control, at hour 4. IL-1RN was increased sharply at hour 1 but quickly reduced to control level at hour 2.

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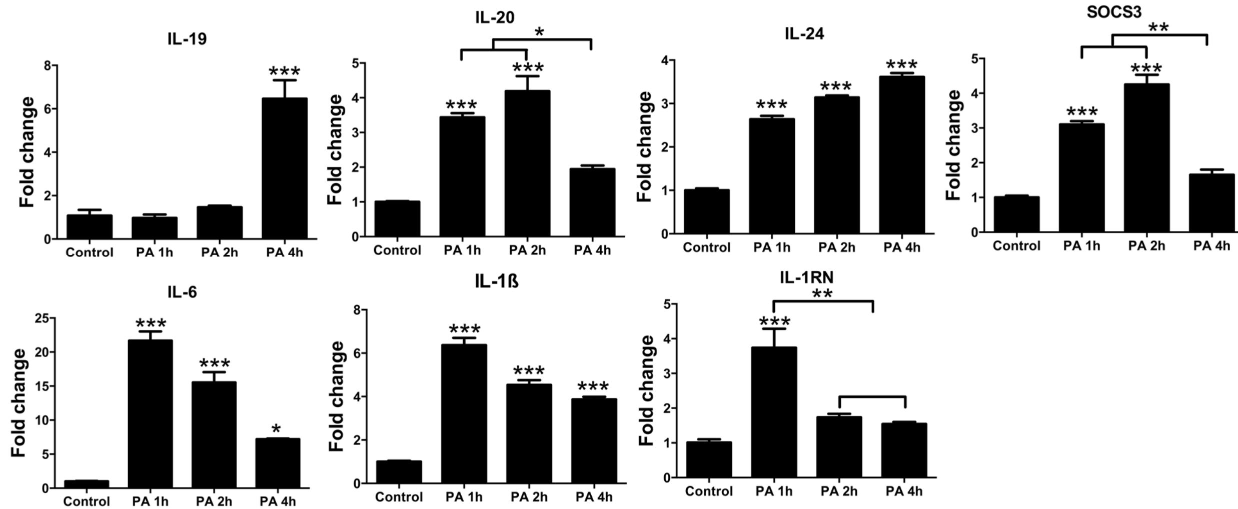

Figure 28 *In vitro*, *PA* challenge induces early expression of IL-24, IL-20 and SOCS3 in primary human corneal epithelial cells (HCECs). Primary HCECs (P3 or P4) were starved overnight, and then challenged with heat-killed *PA* (MOI=50:1) for 1 h, 2h, or 4 h. Cells were subjected to RNA extraction for qPCR analysis of IL-19, IL-20, IL-24, SOCS3, IL-6, IL-1β, and IL-1RN. Results are presented relative to those of unchallenged control cells, set as 1. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (ANOVA). Data are representative of two independent experiments with at least three samples per group (mean+s.e.m.).

IL-24 specifically induces SOCS3 expression in HCECs

Our *in vivo* data demonstrate that both IL-1β and IL-24 are the downstream factors of toll-like receptors. These two cytokines may regulate the expression of each other, as well as other genes involved in the innate defense of the corneas. To this end, we first treated primary HCECs with recombinant human IL-1β and measured cytokine expression (Fig. 29A). The mRNA expression of IL-24 was induced rapidly at hour 1 and remained high at hour 2. IL-1β also induced the expression of SOCS3, IL-1β (self-amplification), and IL-6 (Fig. 29A). The level of SOCS3 mRNA was elevated to 2.2 fold at hour 1 and further elevated to 6.1 fold at 2 hours post IL-1β stimulation. IL-1β induced the mRNA expression of IL-1β with similar levels at hours 1 and 2. IL-6 expression was

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observed to be greater at hour 1 than at hour 2. While significantly elevated IL-19 expression was only seen at 2 h, the expression of IL-20 was induced at hour 1 and further elevated at hour 2. Conversely, recombinant IL-24 stimulated robust upregulation of SOCS3 at hour 1, followed by a drastic decline, yet still significantly higher than the control, at hour 2. IL-24 treatment exhibited no effect on the expression of IL-1β, IL-24, or IL-6, suggesting that IL-24 functions primarily to induce the expression of the negative regulator, SOCS3, in HCECs (Fig. 29B).

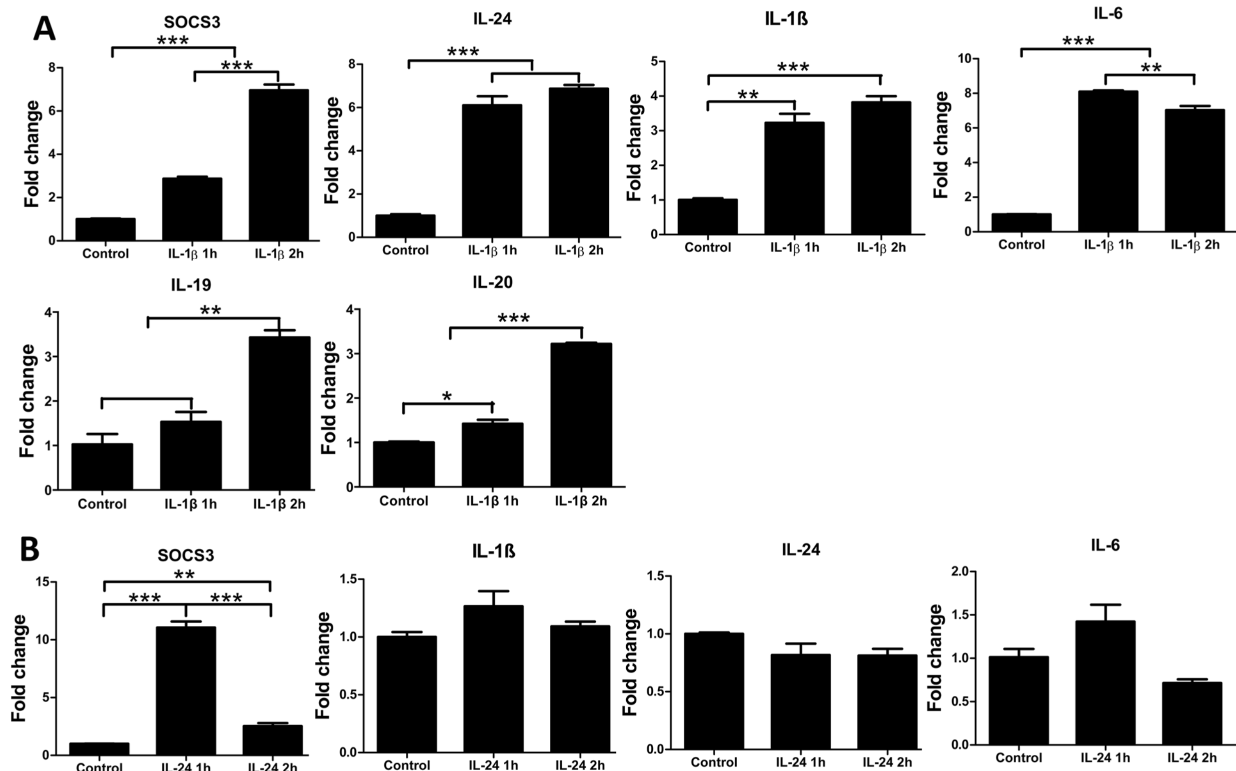

Figure 29 Effects of recombinant human IL-1β or IL-24 on primary human corneal epithelial cells (HCECs). (A) HCECs were treated with recombinant human IL-1β (50ng/ml). Cells were collected at 1h and 2h for qPCR analysis of SOCS3, IL-24, IL-1β, IL-6, IL-19, and IL-20. (B) HCECs were treated with recombinant human IL-24 (100ng/ml). Cells were collected at 1h and 2h for qPCR analysis of SOCS3, IL-1β, IL-24, and IL-6. Results are presented relative to those of untreated control cells, set as 1 (mean+s.e.m.). **P < 0.01, and ***P < 0.001 (ANOVA). Data are representative of three independent experiments.

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Since IL-20 was also elevated at the early time point of *PA* stimulation in primary HCECs *in vitro*, in contrast to no elevation in mouse CECs *in vivo*, we treated HCECs with recombinant human IL-20 to evaluate its effects on cytokine expression. Surprisingly, when we treated the cells with 100ng/ml of recombinant IL-20, the same dose as recombinant IL-24, no expression of SOCS3 was detected by qPCR (Fig. 30A). Then we increased the dosage of recombinant IL-20 to 500ng/ml and found that high dose of recombinant IL-20 activated STAT3 (Fig. 30B and C). The high dose of IL-20, which is unlikely to be the amount produced *in vivo*, induced slight, although significant, elevation of SOCS3 expression (2.2-fold increase) at hour 1 and returned back to control level at hour 2 (Fig. 30D). High dose of recombinant IL-20 failed to induce inflammatory cytokine IL-6 and IL-1β expression in HCECs (Fig. 30D).

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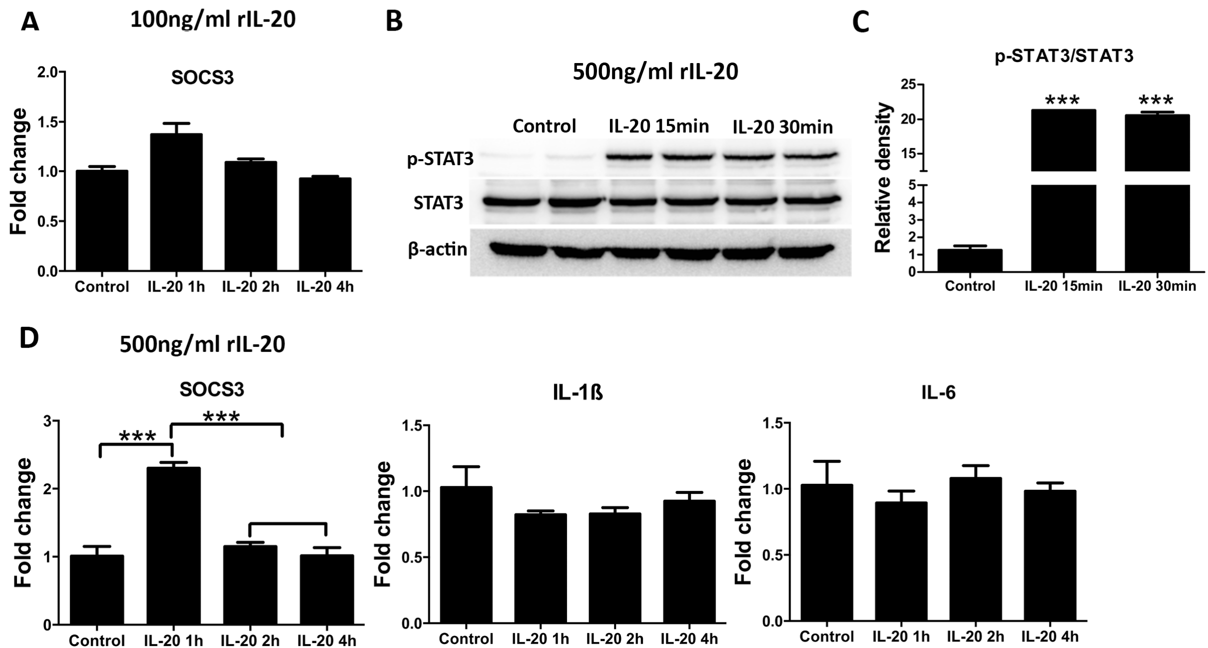

Figure 30 Even high dosage of recombinant IL-20 only slightly induces SOCS3 expression. (A) HCECs were treated with low dosage of recombinant human IL-20 (100ng/ml). Cells were collected at 1h, 2h, and 4h for qPCR analysis of SOCS3. (B) HCECs were treated with high dose of recombinant IL-20 (500ng/ml). Whole cell lysate was collected at 15 min and 30 min to detect the activation of STAT3. β-actin was used as loading control. (C) Quantification of protein levels based on the densitometry of the Western blots in B. (D) HCECs were treated with high dosage of recombinant human IL-20 (500ng/ml). Cells were collected for qPCR analysis of SOCS3, IL-1β, and IL-6. PCR results are presented relative to those of untreated control cells, set as 1 (mean+s.e.m.). Data are representative of two independent experiments.

Discussion

Among the members of IL-20R cytokines, their temporal and spatial specific expression pattern and distinct receptor-ligand interaction may determine their unique biological functions; despite they share the same receptor complexes (Logsdon et al., 2012; Parrish-Novak et al., 2002). IL-19 is not an early responsive gene in response to *PA* infection: elevated expression was first detected at 18hpi in mouse cornea and at 4h

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after challenge in primary HCECs. IL-20, not induced in mouse cornea, was detected in primary HCECs after *PA* challenge. IL-24 readily induces SOCS3 expression in HCECs. However, even large dosage of IL-20 can only slightly induce SOCS3 expression (2.2-fold increase by 500ng/ml IL-20 verses 11.2-fold increase by 100ng/ml IL-24). Although the activity of recombinant proteins awaits to be determined, one explanation is that IL-20 may induce other effects in HCECs. Another explanation is that IL-20 has lower affinity to receptor complex than IL-24. It is reported that only large concentration of anti-IL-20R2 can block IL-24 activity in comparison with IL-20 activity, indicating the lower affinity of IL-20 to the receptors (Sa et al., 2007). In addition, no growth inhibitory effect was detected with IL-20 in ovarian carcinoma cell line comparing to IL-19 or IL-24 even in the same cytokine concentration. The author explained that one possibility is the lower affinity of IL-20 to the receptor complex. When tested at the same concentration, the saturation kinetics are reached for IL-19 and IL-24, but not IL-20 (Parrish-Novak et al., 2002). Since we did not detect elevated expression of IL-1β or IL-6 when the cells were treated with high dosage of recombinant human IL-20, the contamination of LPS in the recombinant protein can be excluded. Therefore, although IL-20R cytokines share the same receptor complex and signaling pathway, they may possess their individual properties and thus foretell their different functions.

IL-1β can induce IL-20R cytokine expression. It is reported that IL-20R cytokines, but not IL-6, suppressed IL-1β production in mouse keratinocytes when challenged with MRSA (Myles et al., 2013). Our data revealed that in HCECs, IL-24 exhibited no effects on IL-1β expression but mediated the expression of SOCS3 in response to infection, suggesting the action of IL-24 may be cell, tissue, and/or pathogen specific. SOCS3

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plays important roles in mounting immune response against pathogens, while suppressing excessive inflammation to avoid damage to the host. However, some pathogens can modify host SOCS3 expression to evade the immune response. Our data suggest that *PA* may have evolved to take advantage of the immunosuppressive property of the IL-24/STAT3/SOCS3 cascade to favor its survival and proliferation in the host; this, however, awaits further investigation. In fact, studies have shown that evolutionarily pathogens have exploited the anti-inflammatory function of IL-10 to facilitate their survival and cause chronic infection (Ouyang et al., 2011).

In sum, our study demonstrated that heat-killed *PA* challenge induced early IL-24 and IL-20 expression, and late IL-19 expression in primary HCECs. IL-24 induced robust SOCS3 expression, but not the expression of the inflammatory cytokines often associated with microbial keratitis. The readiness of IL-24 inducing the negative regulator expression highlights its anti-inflammatory property.

OVERALL SUMMARY

The present study demonstrated that among the three IL-20R cytokines, only IL-24 was markedly induced at 3 hpi, followed by a great decline and a gradual increase; while IL-1β expression was steadily increased during the course of infection. Interestingly, SOCS3 shared a similar expression pattern with IL-24; while the expression of another STAT3 activator, IL-6, was not increased until 9 hpi. Functional studies revealed that while exogenous IL-24 increased the severity of *PA* keratitis, the downregulation of the IL-24 signaling pathway resulted in reduced clinical scores, less opacity, lower bacterial burden, and reduced infiltration of neutrophils. At the early stage of infection (6 hpi) when most invading pathogens remained at the epithelial layer,

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downregulation of IL-24 markedly increased the infection-induced expression of AMPs S100A8 and A9; while the expression of IL-1β, its natural inhibitor, IL-1RN, MMP13, and SOCS3 was decreased. At 1 dpi, the expression of the inflammatory cytokines, (IL-1β, IL-6, IL-17A, pathogenic factor MMP13), S100A8/A9, CRAMP, and SOCS3 was all markedly elevated in infected corneas. Elevations in expression were repressed to different extents by IL-24 downregulation. We also demonstrated that IL-24 was a major inducer of the activation of STAT3, and the expression of its negative regulator, SOCS3, at the early stages of infection. SOCS3 was shown to be required for controlling the progress of *PA* keratitis. *In vitro* study using cultured HCECs revealed that while IL-1β induced the expression of a battery of cytokines, including itself and SOCS3, IL-24 induced only the expression of SOCS3. Taken together, our data suggest that IL-24 is an early response gene involved in corneal innate immune defense and inflammation. The induction of SOCS3 at early stages of infection by IL-20R cytokines may interfere with the inflammatory response necessary for effective innate immune defense against microbial infection. This raises the concern for the use of IL-24 as an anticancer agent, because it may render patients more susceptible to opportunistic infection at a time when their immune systems are already comprised. In the cornea, downregulation of IL-24 may be used as an effective mean to boost innate immunity, and hence to reduce the rate of infection by opportunistic ocular pathogens such as *PA.*

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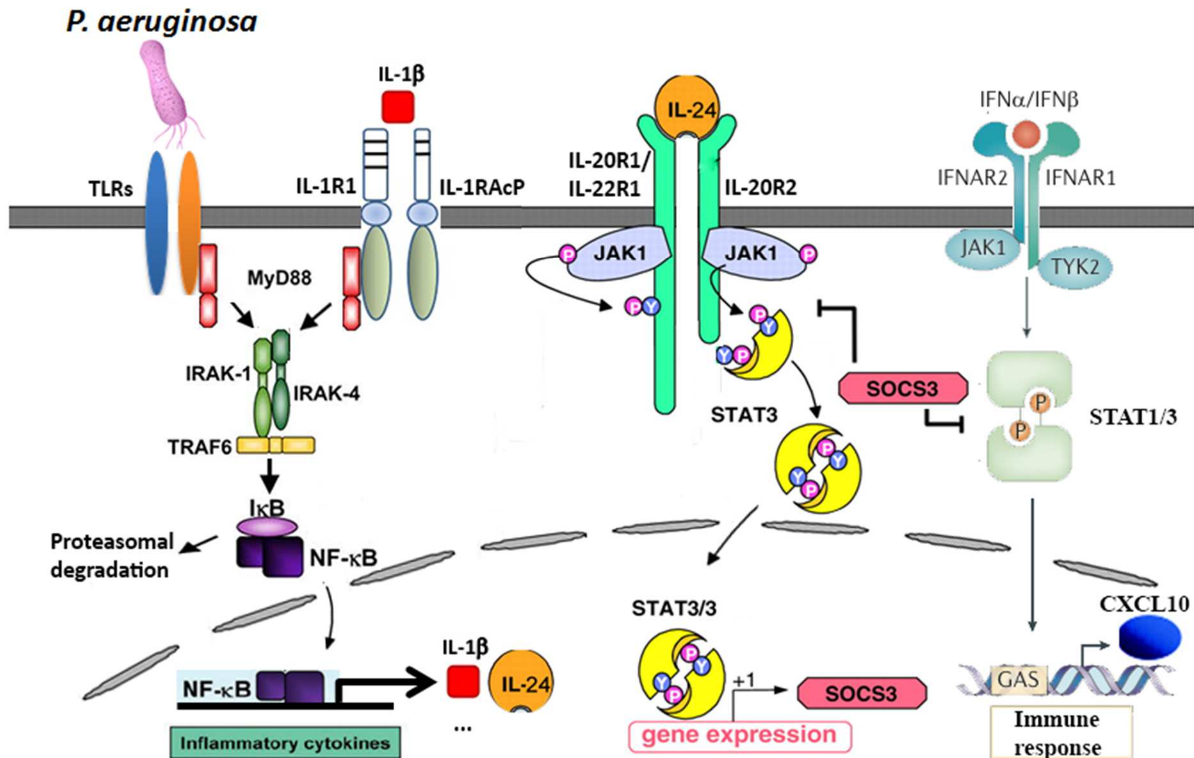

Figure 31 Schematic graph summarizing part of the signaling pathways involved in IL-24/STAT3/SOCS3 signaling events. In summary, in the case of *PA* infection, the surface components of *PA,* such as flagellin and LPS, are sensed by TLRs in corneal epithelial cells. Signals are transduced by MyD88 and NF-κB pathway to induce IL-24 and inflammatory cytokine expression. IL-24, by binding to its heterodimer receptors in corneal epithelial cells, activates JAK/STAT3 signaling pathway to induce early expression of the negative regulator SOCS3. SOCS3 negatively regulates STAT protein activation, including STAT1 and STAT3 to suppress the necessary inflammatory response. Interferon signaling pathway plays an important role in both bacterial and fungal infection. By activating STAT1/STAT3, interferon can regulate CXCL10 expression, which processes direct antimicrobial activity. When silencing of IL-24, interferon pathway is activated with less restriction of negative regulator SOCS3; therefore, more CXCL10 is produced to clear the pathogens in corneal epithelial cells, resulting in less keratitis seen in IL-24 silencing group. SOCS3 is an important negative regulator, but what matters is the timing of its expression. We believe that trying to shut down the necessary immune response at an early time point is detrimental to the host to control infection. As infection and inflammation continue, inflammatory cytokine IL-1β may further induce IL-24 expression, which cause a cascade of inflammation and is detrimental to the host.

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MATERIALS AND METHODS

Animals

Wild-type C57BL/6 mice (8 weeks, female) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). MyD88 knockout mice were kindly provided by Dr. Ashok Kumar. The knockout mice were bred in our Institutional Animal Care Facility and genotyped before use to ensure their gene deficiency. All animal procedures were performed in compliance with the ARVO Statement on the use of animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Wayne State University.

Flagellin preparation

Flagellin was prepared from *PA* strain *PAO1* as previously reported (Gao et al., 2010; Zhang et al., 2003). Briefly, flagellin was isolated from *PAO1* by ammonium sulfate precipitation, followed by DEAE-Sephadex A-50 chromatography. LPS was removed by using Detoxi-Gel Affinity Pak columns (Pierce, Rockford, IL). A quantitative Limulus amebocyte lysate kit was used to detect the amount of LPS in the flagellin samples. After the two chromatography steps, LPS level was 0.0027 endotoxin unit/μg protein. By this method, up to 1,000 ng/ml purified flagellin was unable to stimulate HeLa cells with functional TLR4, CD14, and MD2 (Re and Strominger, 2002), which are otherwise activated by 1 ng/ml purified *PA* LPS (Sigma, St. Louis, MO, USA).

Bacterial strain

P. aeruginosa strain *ATCC 19660* was used in this study. *ATCC 19660* is the most virulent strain. It can cause keratitis in B6 mouse at 1.0 x 10^4 CFUs/cornea. *PA* was maintained on tryptic soy (Sigma-aldrich, St. Louis, MO, USA) agar plates at 4 °C.

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For use in experiments, a colony of *PA* from the tryptic soy agar plate was cultured in liquid tryptic soy broth medium at 37 °C overnight under shaking. *PA* were harvested by centrifugation (5000 rpm for 5 min) and resuspended in sterile PBS. According to a predertermined OD_{600} conversion factor: OD_{600} =0.5 corresponding to 1×10^8 CFUs/ml bacteria, dilute the bacteria with PBS to yield 2×10^6 CFUs/ml bacteria for the *in vivo* studies. (OD_{600} is measured at Spectrophotometer, Eppendorf Biophotometer D30, Germany)

Mouse model of *P. aeruginosa* Keratitis

Mice were anesthetized with an intraperitoneal injection of Ketamine (90 mg/kg) and Xylazine (10 mg/kg) before surgical procedures. Mouse corneas were scratched gently with a sterile 26-gauge needle to create three 1-mm incisions to break the epithelial barrier. Purified flagellin (500ng in 5μl PBS) was applied topically to the injured cornea as an eye drop. PBS was used as control. Twenty-four hours later, the corneas were scratched again and inoculated with 1.0×10^4 CFUs of *ATCC 19660* in 5μl PBS.

The application of siRNA, recombinant protein, or neutralizing antibody

All the siRNAs used in this study were SMARTpool (a mixture of 4 siRNAs) ON-TARGETplus siRNAs designed by GE Dharmacon Company (Lafayette, CO, USA). Mice were subconjunctivally injected twice with siRNA targeting to a specific gene (10μM/ul, 5μl) over 2 days. Six hours after the second siRNA injection, mouse corneas were inoculated with *PA* to observe the silencing effects of the specific gene on infection. To apply recombinant protein or neutralizing antibody, mice were subconjuntivally injected with recombinant mouse IL-24 (200ng/5ul, 7807-ML-010; R&D

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systems, Minneapolis, MN, USA), or anti-IL-20R2 (200ng/5ul, 14-1206; eBioscience, San Diego, CA, USA) 4 hours before the inoculation with *PA* on the corneas.

Clinical examination, quantification of *PA* colony forming units (CFUs) and myeloperoxidase (MPO) units

The mice were monitored and the corneas were photographed daily up to 3dpi for the assessment of infection severity. Clinical scores were assigned to the infected corneas in a blind fashion according to the scale previously reported (Wu et al., 2003) (Table 4). A total possible score ranging from 0 to 12 will be designated to an infected cornea. A total score ≤ 5 is categorized as mild, 6-9 as moderate, and ≥9 as severe infection.

Table 4 Visual clinical scoring system for murine keratitis. (Wu et al., 2003)

	Grade 1	Grade 2	Grade 3	Grade 4
Area of corneal opacity	1%–25%	26%–50%	51%–75%	76%–100%
Density of corneal opacity	Slight cloudiness, outline of iris and pupil discernable	Cloudy, but outline of iris and pupil remain visible	Cloudy, opacity not uniform	Uniform opacity
Surface regularity	Slight surface irregularity	Rough surface, some swelling	Significant swelling, crater or serious descemetocele formation	Perforation or descemetocele

Whole corneas were excised from the eyes and put in 200μl sterile PBS. Tissue was homogenized with TissueLyser II (Qiagen, Valencia, CA, USA) at 30Hz for 3 min. The homogenates were divided into two parts. The first part (50μl) was subjected to serial log dilutions and then plated onto TS agar plate in duplicate for the assessment of bacterial viability. Following overnight incubation at 37 °C, the number of CFUs was determined by direct counting. The remaining homogenates was further lysed with the lysate buffer, 1% SDS and 10% Triton X-100 in PBS (5μl for 50μl sample).

For MPO measurement, 60μl of homogenates were put into 240μl of hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50mM potassium

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phosphate buffer, pH=6.0). The samples underwent four freeze-thaw cycles, followed by sonication on ice (3 sec × 3 times) (Sonic Dismembrator model 100, Fisher Scientific, Waltham, MA, USA). Subsequently, the samples were centrifuged at 14,000 rpm for 30 min to obtain supernatant. In a 96-well plate, 20μl of supernatant was mixed with 180μl phosphate buffer (50mM, pH=6.0) containing O,O-dianisidine hydrochloride (16.7 mg/100mL) and 0.0005% hydrogen peroxide. MPO units were measured immediately in absorbance at 460 nm continuously for 5 min with a microplate reader (Synergy2; BioTek, Winooski, VT, USA). The results were expressed in MPO units per cornea. 1 MPO unit corresponds to 2.0×10^5 polymorphonuclear leucocytes (PMNs) (Williams et al., 1982).

Semi-quantitative and quantitative PCR

Gene specific primers were designed by using online Primer3 software system and purchased from Integrated DNA Technologies (IDT), Inc. (Coralville, Iowa, USA). The primers used in this study are listed in Table 5. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. RNA was reversed-transcribed to cDNA with a first-strand synthesis system (Derkin Elmer Letus, DNA Thermal Cycler 480, Norwalk, CT, USA). For semi-quantitative PCR, cDNA was amplified with TaqMan technology (Promega, Madison, WI, USA). PCR products were subjected to electrophoresis on 2% agarose gels containing ethidium bromide. GAPDH was used as loading control. Stained gels were captured with a digital camera under UV transillumination.

For quantitative PCR, cDNA was amplified using StepOnePlus Real-Time PCR system (Applied Biosystems, University Park, IL, USA) with the SYBR® Green PCR

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Master Mix (Applied Biosystems). Melting curve and dissociation curve analyses were used to confirm the utility of each pair primers and ensure only the desire product was amplified, respectively. Data were analyzed by using ΔΔCT method with β-actin or GAPDH as the internal control. Relative mRNA levels in each group were obtained from the means of three or four biological independent experiments.

Table 5 PCR primer sequences used in this study.

Primers	Forward	Reverse
mIL-19	aacaacctgctgacattcta	attggtggcttcctgact
mIL-20	tctcagtctgtcattctcac	tatagcatctcctccatcca
mIL-22	cacttgtgcgatctctga	gatgtatggctgctggaa
mIL-24	gctttcaccaaagcgacttc	gcccagtaaggacaattcca
mSOCS3	attcacccaggtggctacag	gccaatgtcttcccagtgtt
mSOCS1	acttctggctggagacctca	cccagacacaagctgctac
mIL-1β	tgccaccttttgacagtgatg	aaggtccacgggaaagacac
mIL-1RN	gggaccctacagtcacctaa	ggtccttgtaagtacccagca
mIL-6	gtggctaaggaccaagacca	taacgcactaggtttgccga
mMMP13	tgatgaaacctggacaagc	ctggaccataaagaaactgaa
mIL-17A	tttaactcccttggcgcaaaa	ctttccctccgcattgacac
mS100A8	ttcgtgacaatgccgtctga	agggcatggtgatttccttgt
mS100A9	tgggcttacactgctcttacc	ggttatgctgcgctccatct
mCRAMP	gtcttgggaaccatgcagtt	caggtccaggagacggtag
mIL-20R1	aatgcgggagtatcaacagg	gatagaagcgttccgtctcg
mIL-20R2	caaagagatgctccgaggac	cctcccagacaccttgaaaa
mIL-22R1	aaggtcaggacacgttggac	tgctcttttccatgggtttc
mβ-actin	gtgggccgccctaggcacca	ggttggccttagggttcagg
hIL-19	tacgtggacagggtgttcaa	acattgccgcagagttttct
hIL-20	ctcaggcagcagttgtgaag	agcagcagcatcactttcct
hIL-24	gtctttcacagcccagaagg	agggccaagaattccacttt
hSOCS3	ggtaccccacatcctctcct	caaatgttgcttccccctta
hIL-6	tttcaccaggcaagtctcct	gaaagcagcaaagaggcact
hIL-1β	accaaacctcttcgaggcac	agccatcatttcactggcga
hGAPDH	aggggtctacatggcaactg	ggcctccaaggagtaagacc

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ELISA

For cytokine measurement, homogenates from mouse cornea samples were sonicated and centrifuged to obtain supernatant. Protein concentration was determined by BCA assay with Thermo Scientific Pierce BCA Protein Assay Kit (Micro BCA, Pierce, Rockford, IL, USA). The amount of cytokines was determined by ELISA assay according to manufacturer's instructions (mouse IL-1β: DY401; mouse IL-1RA: MRA00; mouse IL-6: DY406; mouse S100A8/9: DY8596-05; R&D Systems, Minneapolis, MN, USA).

Western Blot

Mouse corneal samples were lysed with RIPA buffer. The lysates were centrifuged to obtain supernatant. Protein concentration was determined by BCA assay. The protein samples were separated by SDS-PAGE and electrically transferred onto nitrocellulose membranes (Bio-Rad; Hercules, CA, USA). The membranes were stained with 2% Ponceau S. to visualize all proteins. According to prestained protein molecular size marker and the molecular weights of interested proteins, the membranes were cut into strips. Then the strips were blocked with 3% BSA for at least 1h at room temperature, followed by incubation with primary antibodies overnight at 4°C. After several washes with Tris-buffered saline/0.05%Tween 20 (TBST), the strips were incubated with HRP-conjugated secondary antibodies. Signals were visualized using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Pittsburgh, PA, USA). β-actin was used as the loading control. Antibodies: anti-IL-24 (ab182567; Abcam, Cambridge, MA, USA); anti-SOCS3 (#2923; Cell signaling, Danvers, MA, USA);

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anti-STAT3 (#4904; Cell signaling); anti-phospho-STAT3 (Tyr705) (#9145; Cell signaling); and anti-β-actin (A1978; Sigma).

STAT3 has two isoforms: STAT3α (86 kDa) and STAT3β (79 kDa). Their relative expression levels depend on ligands, cell types, and maturation state of cells (Biethahn et al., 1999). Therefore, Western blot shows two bands for both p-STAT3 and STAT3. Quantification of protein levels was based on the densitometry of blots by using the software Carestream MI SE (Informer Technologies the Western blots, Inc.). Rectangles were drawn to include the bands manually. When the target protein has two bands, both two bands would be included in the rectangle. Analyze the bands to get net intensity. The target protein would first compare with the control in the same group and then normalize with β-actin to get a relative density. The results were plotted as mean+s.e.m. to get a bar graph.

Primary human corneal epithelial cell culture

Primary human corneal epithelial cells (HCECs) were obtained from diseased human corneal samples. Corneal epithelial cells were dislodged from underlying basement membrane by dispase and then digested by trypsin. Cells were pelleted, re-suspended and grown in defined keratinocyte serum-free medium (DK-SFM) (Gibco, Waltham, MA USA) with supplements and antibiotics (penicillin and streptomycin).

P3 or P4 of the primary HCECs were used for the experiments. Before treatment, cells were starved overnight in growth factor-free and antibiotic-free keratinocyte basic medium (KBM-2, Lonza, Basel, Switzerland). Subsequently, cells were challenged with heat-killed *ATCC* with MOI=100:1 (MOI was determined according to previous report (Ni et al., 2008)) or treated with recombinant human IL-1β (50ng/ml, 201-LB; R&D systems,

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Minneapolis, MN, USA) (Dosage was determined according to previous report (Cella et al., 2010)), recombinant human IL-24 (100ng/ml, 1965-IL-025; R&D systems), or recombinant human IL-20 (100ng/ml or 500ng/ml, 1102-IL-025; R&D systems) (The dosage of both IL-24 and IL-20 was determined according to previous report (Kreis et al., 2007)). At the end of the incubation period, cells were harvested to detect gene expression. Time points were determined according to previous report (Zhang et al., 2009).

Primary mouse bone marrow-derived macrophage (BMDM) culture

Wild-type C57BL/6 mice (8 weeks) were euthanized by rapid cervical dislocation. Using aseptic technique, femur was dissected out and sterilized with gentamycin in PBS (20ug/ml). Bone marrow was flushed with RPMI-1640 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 mM 2-mercaptoethanol. Red blood cells (RBC) were lysed with RBC Lysis Buffer. Cells were plated on non-tissue culture treated petri dishes and differentiated into macrophages in the presence of mouse recombinant macrophage colony stimulating factors (M-CSF, 20 ng/ml; PeproTech, Rocky Hill, NJ). After 7 days of differentiation period, BMDM were used for experiments.

The efficiency of the differentiation was assessed using Flow Cytometry analysis of Mac-1 and F4/80 surface antigen expression. Cells were washed with PBS and harvested by trypsinization, followed by incubation with Fc Block (anti-mouse CD16/32, 1:100 dilution, BD, San Jose, CA) for 20 min at room temperature. Then cells were washed incubated with FITC-conjugated anti-F4/80 (1:100 dilution, BD) and PE-conjugated anti-Mac-1 (1:100 dilution, BD) antibodies for 30 min in the dark. Cells were

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washed and resuspended with 2% FBS in PBS. Flow cytometry was used to analyze the cells. Macrophages are double-positive for F4/80 and Mac-1.

For experiments, BMDMs were plated into 12-well plates. The next day, cells were starved with RPMI 1640 medium supplemented with 1% FBS for 1h. Then BMDMs were treated with either heat-killed *PA* (MOI=50) for 2h. Cells were collected for western blot analysis.

Statistical analysis

A nonparametric Mann-Whitney U test was used to compare the clinical scores. Student t-test or one-way ANOVA was used to compare quantitative means. P-value < 0.05 was considered to be significant.

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ABSTRACT

EXPRESSION AND FUNCTIONS OF IL-24 AND SOCS3 IN *PSEUDOMONAS AERUGINOSA* KERATITIS IN A C57BL/6 MOUSE MODEL

by

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August 2016

Advisor: Dr. Fu-shin Yu

Major: Anatomy and Cell Biology

Degree: Doctor of Philosophy

The aim of this study was to elucidate the expression and functions of interleukin (IL)-24 and suppressor of cytokine signaling 3 (SOCS3), and their regulatory relationship in C57BL/6 mouse corneas in response to *Pseudomonas aeruginosa (PA)* infection. Among IL-20R cytokines, only IL-24 was induced at both mRNA and protein levels by the infection, and this upregulation was dampened by flagellin pretreatment. Time course studies revealed that IL-24 expression was markedly elevated, followed by a subsidence and second elevation, a pattern shared with SOCS3 but not IL-1β or IL-6. Silencing of IL-24 enhanced S100A8/A9 expression, and suppressed SOCS3, IL-1β, IL-1RN, and MMP13 expression during an early stage of infection. Downregulation of IL-24 signaling pathway significantly reduced the severity of keratitis, bacterial burden, and neutrophil infiltration; while recombinant IL-24 exacerbated *PA* keratitis. Furthermore, SOCS3 knockdown impaired the control of *PA* keratitis. *In vitro*, while IL-1β induced the expression of SOCS3, IL-24, IL-1β, and IL-6, IL-24 only elicited robust expression of SOCS3 in primarily cultured human corneal epithelial cells. In conclusion, IL-24 promotes *PA* keratitis by inducing SOCS3 expression, resulting in the suppression of

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the necessary inflammatory response at an early stage of infection, and in the increased severity of *PA* keratitis.

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- Extraction of protein and quantification with Western Blotting or ELISA
- Immunohistological staining and quantitative histological analysis
- Culture, isolation, and preservation of bacterial and fungal stains
- *In vivo* animal studies: skilled in mouse surgery under microscope
- Primary cell isolation and culture

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